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712357

Union Carbide Corporation
Nuclear Division
ATTN: Dr. Herman Postma, Director
Oak Ridge National Laboratory
Post Office Box X
Oak Ridge, Tennessee 37830

Gentlemen:

ORNL PROJECT ENTITLED "RETROVIRAL GENETIC EXPRESSION IN HUMAN CANCERS:
ANALYSIS BY PRIMER tRNA BINDING APPROACH" (INTERAGENCY AGREEMENT DOE
NO. 40-699-78, NCI NO. Y01 CP 90503)

The Department of Energy and the National Cancer Institute have executed
the enclosed subject agreement covering Dr. W. R. Yang's research project
as described in the proposal transmitted by your letter of July 24, 1978.
This is a three-year project for which NCI has funded the first eight months
through September 30, 1979, in the amount of \$113,070.

You are authorized to conduct the project for EPA in accordance with the
proposal and the enclosed agreement. The \$113,070 authorized by this letter
will be included in the UCCND contract for reimbursable work under Activity
40 04 04. This work is of programmatic interest to DOE and shall be conducted
on an actual cost basis, depreciation and DOE added factor waived.

Please confirm your willingness to conduct this new work for NCI as incidental
or related to the approved DOE program at ORNL pursuant to Article III,
Paragraph (c) of Contract No. W-7405-eng-26 by signing and dating a copy
of this letter and returning it to us.

Sincerely, Richard L. Egan

Joseph A. Lenhard, Assistant Manager
for Energy Research and Development

ER-13:WRB

Enclosure:
Agreement

- cc w/encl:
- R. F. Hibbs, UCCND
- G. W. Horde, UCCND
- J. K. Denton, UCCND
- W. T. Carter, UCCND
- C. W. Hill, ORO
- H. S. Oster, ORO
- T. W. White (2), ORO

1 26/3

ACCEPTED BY S. Herman Postma

DATE: 4/2/79

ER-13:WRBibb:ipc:6-0742:3-19-79
OFFICIAL FILE COPY

CONCURRENCES
RTG. SYMBOL AD-41 White
INITIALS/SIG.
DATE
RTG. SYMBOL ER-10 Lenhard
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RTG. SYMBOL ER-13 Bibb
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DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NATIONAL INSTITUTES OF HEALTH
BETHESDA, MARYLAND 20814

NATIONAL CANCER INSTITUTE

February 15, 1979

Our Reference: Contract No. N01 CP 90503

Department of Energy
Oak Ridge Operations
P. O. Box E
Oak Ridge, Tennessee 37830

Attention: Mr. Joseph A. Lenhard, Director
Research and Technical Support Division

Dear Mr. Lenhard:

We are enclosing an executed copy of the subject agreement for your retention. If you have any questions regarding its administration, please contact the undersigned.

Sincerely yours,

J. Thomas Lewin
Contracting Officer
VOFSCS, RCB, OD, NCI
Landow Building, Room C409
7910 Woodmont Avenue
NIH, Bethesda, Maryland 20014

Enclosure

46-10
MAR 02 1979

Y 1831

1078796

NATIONAL INSTITUTE OF HEALTH
INTERAGENCY AGREEMENT

NEGOTIATED PURSUANT TO
Economy Act of 1932, as
amended (31 USC 686)

TYPE OF Agreement
Cost Reimbursement

261790503

ISSUING OFFICE

National Cancer Institute
VOFSCS, RCB, OD
Landow Building, Room 4C09
7910 Woodmont Avenue
NIH, Bethesda, Maryland 20014

Agreement For

Retroviral Genetic Expression in Human Cancer:
Analysis by Primer tRNA Binding Approach

Agency (Name and Address):

Department of Energy
Oak Ridge Operations
P. O. Box E
Oak Ridge, Tennessee 37830

ACCOUNTING AND APPROPRIATION DATA

CAN 9-8422564(\$113,070)
Subobject Class 25.1L
Document No. Y1CP90503A
EIN: 1-362165429-A1

PLACE OF PERFORMANCE

Oak Ridge, Tennessee

CONTRACT AMOUNT

\$113,070.00

MAIL VOUCHERS TO

See Billing Instructions (Article IV)

SPONSOR

Division of Cancer Cause & Prevention,
Biological Carcinogenesis

EFFECTIVE DATE

February 1, 1979

EXPIRATION DATE

September 30, 1979

The Agency agrees to furnish and deliver all the supplies and perform all the services set forth in the attached Special Provisions, for the consideration stated herein. The rights and obligations of the parties of this Agreement shall be subject to and governed by the Special Provisions. To the extent of any inconsistency between the Special Provisions and any specifications or other provisions which are made a part of this contract, by reference or otherwise, the Special Provisions shall control.

IN WITNESS THEREOF, the parties hereto have executed this Agreement on the day and year last specified below.

Department of Energy	National Cancer Institute, NIH
NAME OF Agency	
BY 	BY 
SIGNATURE OF AUTHORIZED INDIVIDUAL	SIGNATURE OF CONTRACTING OFFICER
K. M. Haythorn, Director	J. Thomas Lewin
Energy Programs and Support Division	TYPED NAME
FEB 13 1979	February 15, 1979
TITLE	DATE
DATE	

THIS CONTRACT CONSISTS OF:

- 1. COVER PAGE HEW-554
- 2. CONTENTS OF CONTRACT HEW-555
- 3. SPECIAL PROVISIONS HEW-556

ARTICLE	I	DESCRIPTION OF WORK
ARTICLE	II	PERIOD OF PERFORMANCE
ARTICLE	III	CONSIDERATION
ARTICLE	IV	BILLING PROCEDURES
ARTICLE	V	PROJECT OFFICER
ARTICLE	VI	KEY PERSONNEL

ARTICLE I
DESCRIPTION OF WORK

- A. The Department of Energy (hereinafter referred to as the "Agency") shall furnish services, qualified personnel, and material, equipment, and facilities, not otherwise provided by the National Cancer Institute under the terms of this agreement, as needed to perform the work set forth below.
1. Isolation of poly(A) RNAs from various human normal and cancer cells; quantitative and qualitative comparison of their capacity to bind selective tRNA species; assessment of the primer property of the bound tRNAs by reverse transcription reaction; and determination of whether different types of human cancers contain poly(A) RNA of different tRNA binding specificity.
 2. Structure analysis of tRNA poly(A) RNA binding nucleotide sequences as well as reversely transcribable 5' end sequences of the tRNA-binding site in the poly(A)RNA, by employing nucleotide sequence determination and molecular hybridization.
 3. Purification of the specific poly(A)RNAs by using the unique "primer tRNA" binding specificity.
 4. Characterization of the specific poly(A)RNAs, including messenger RNA activity by protein synthesis assay and possible changes of level during neoplastic process.
- B. Reporting Requirements
1. The Agency shall submit an annual progress report four months before the anniversary date of the agreement, including a short summary (NIH-2198), a narrative description by task of work accomplished and a statement of the importance of the findings.
 2. The Agency shall also submit semiannually (2 months after the anniversary date) a brief summary of progress (NIH-2198) during the previous six months. A final report will be due 30 days after completion or termination of the agreement.
 3. Send all progress reports to the National Institutes of Health, NCI, Biological Carcinogenesis Branch Information Unit, Landow Building, Room 9A22, Bethesda, Maryland 20014. A reminder will be sent by the Unit specifying the number of copies required and date report is due.
 4. In all publications resulting from research performed under the agreement, the Agency shall acknowledge support of the Division of Cancer Cause and Prevention, National Cancer Institute, NIH, DHEW, under Agreement No. Y01 CP 90503.

5. Manpower Report

The Agency shall complete an annual Manpower Report, form NIH-1749 (Rev. 1/77), in accordance with the instructions contained therein. The parties agree that this report shall not be used for any reconciliations with other fiscal reports or for any other fiscal or accounting purposes.

- C. All work performed under this agreement must be conducted in accordance with the National Cancer Institute Safety Standards for Research Involving Oncogenic Viruses [DHEW Publication No. (NIH) 75-790] and National Cancer Institute Safety Standards for Research Involving Chemical Carcinogens [DHEW Publication No. (NIH) 76-900].

ARTICLE II
PERIOD OF PERFORMANCE

The period of performance of this agreement shall be from February 1, 1979, through September 30, 1979.

ARTICLE III
CONSIDERATION

1. The presently estimated cost of the work under this agreement is \$113,070.00.

ARTICLE IV
BILLING PROCEDURES

The Agency shall submit vouchers for cost reimbursement on Government Standard Form 1080 (Voucher for Transfers Between Appropriations and/or Funds) on a quarterly basis. An original and five (5) copies of all such vouchers shall be sent to the following address:

Contracting Officer
Viral Oncology and Field Studies Contracts Section
Research Contracts Branch
National Cancer Institute
Landow Building, Room 4C17
7910 Woodmont Avenue
NIH, Bethesda, Maryland 20014

ARTICLE V
PROJECT OFFICER

The following Project Officer will represent the National Cancer Institute for the purpose of this agreement:

Dr. Charles J. Sherr

The Project Officer is responsible for: (1) Monitoring the Agency's technical progress, including the surveillance and assessment of performance and recommending to the Contracting Officer changes in requirements; (2) interpreting scope of work; (3) performing technical evaluation as required; (4) performing technical inspections and acceptances required by this agreement; and (5) assisting the agency in the resolution of technical problems encountered during performance. The Contracting Officer is responsible for directing or negotiating any changes in terms, conditions, or amounts cited in the agreement.

ARTICLE VI
KEY PERSONNEL

The individual(s) cited below are considered to be key personnel essential to the work and services to be performed under this Agreement and in the event that such personnel leave the Agency's employ or are reassigned to other programs, the Agency shall notify the National Cancer Institute's Contracting Officer reasonably in advance, and shall submit their proposed replacement(s) with qualifications, in sufficient detail to permit evaluation of the impact on the program. No diversion or replacement shall be made by the Agency without the written consent of the Contracting Officer.

Wen K. Yang, M.D., Ph.D.

Principal Investigator

OAK RIDGE NATIONAL LABORATORY

OPERATED BY
UNION CARBIDE CORPORATION
NUCLEAR DIVISION



POST OFFICE BOX X
OAK RIDGE, TENNESSEE 37830

July 24, 1978

OFFICE OF THE DIRECTOR

Department of Energy
Oak Ridge Operations
Attention: Mr. J. A. Lenhard, Director
Research and Technical Support Division
Post Office Box E
Oak Ridge, Tennessee 37830

50-200-1187

Gentlemen:

Interagency Agreement Proposal to NCI
"Retroviral Genetic Expression in Human Cancers"

Enclosed for your review and approval is an Interagency Agreement research proposal being submitted to the National Cancer Institute by Dr. W. K. Yang, Biology Division. This proposal requests a three year performance with total funding in the amount of \$593,200, with \$169,600 required for the initial twelve-month period.

It is requested that this proposal be exempt from any added cost factors because of the programmatic relation with DOE Activity GK 01 02 02 1, "Health Effects Research in Biological Systems - Carcinogenesis."

After your approval and approval of the Division of Biomedical and Environmental Research, please forward forty (40) copies of the technical proposal and five (5) copies of the business proposal to Mr. Charles Fafard, Chief Contract Specialist, Research Contract Branch, Viral Oncology Program, National Cancer Institute, Landow Building, Room 9A-04, Woodmont Avenue, Bethesda, Maryland 20014.

If you have any questions relating to this proposal, please contact C. R. Richmond, Ext. 3-1477.

Sincerely yours,

Herman Postma
Director

HP:VBI:nt
Enclosure

cc: R. J. M. Fry
R. F. Hibbs
W. R. Ragland

C. R. Richmond
J. B. Storer
File - RC

JUL 27 1978

1078802

0-11-15-50
40-6-79

Business Proposal

NCI-DOE Cancer Research Program

Retroviral Genetic Expression in Human Cancers:
Analysis by Primer tRNA Binding Approach

Principal Investigator: Wen K. Yang, M.D., Ph.D.
Biology Division, Oak Ridge National Laboratory
Oak Ridge, Tennessee

Proposed Addition to Interagency Agreement:
Department of Energy and
National Cancer Institute

Proposed Period - October 1, 1978 - September 30, 1981

1078803

PROPOSED BUDGET

Proposed Addition to NCI-DOE Interagency Agreement

No. Y01 CP 60500

"Retroviral Genetic Expression in Human Cancers: Analysis by
Primer tRNA Binding" (Yang)

	FY 1979		FY 1980		FY 1981	
	MY	\$	MY	\$	MY	\$
W. K. Yang	0.25		0.50		0.50	
D. L. Hwang	<u>1.00</u>		<u>1.00</u>		<u>1.00</u>	
Total Ph.D.	1.25		1.50		1.50	
D. J. Price	1.00		1.00		1.00	
C. D. Stringer	0.50		0.50		0.50	
D. M. Yang	<u>0.50</u>		<u>0.50</u>		<u>0.50</u>	
Total Technicians	2.00		2.00		2.00	
DIRECT COSTS:						
Salaries		59,200		67,700		71,700
Fringe Benefits		15,500		17,600		19,300
Materials & Supplies		17,000		26,000		30,000
Travel		1,200		1,200		1,200
Shop & Maintenance		800		1,200		1,200
Glassware Washing		<u>6,000</u>		<u>7,000</u>		<u>7,700</u>
Total Direct Costs	3.25	99,700	3.50	120,700	3.50	131,100
INDIRECT COSTS:						
Division Administration		16,600		19,600		20,800
Utilities		12,000		13,000		14,500
ORNL General & Administrative		<u>41,300</u>		<u>49,100</u>		<u>54,800</u>
Total Indirect Costs		69,900		81,700		90,100
TOTAL		<u>\$169,600</u>		<u>\$202,400</u>		<u>\$221,200</u>

1078804

COST ELEMENTS

- a. Fringe Benefits. 26% of direct salaries.
- b. Division Administration. Includes administrative and central costs associated with management of the Biology Division such as secretarial, purchasing, editorial, financial, library, radiation safety and monitoring, and general maintenance items. 28% of direct salaries.
- c. ORNL G+A. Supports general staff of ORNL such as management offices, personnel, wage and salary, health services, fire and security departments, stores, receiving, accounting and other central services and staff functions. 31% of total costs.
- d. Travel. Funds are requested to support travel of each investigator to at least one scientific meeting (American Association for Cancer Research, American Society of Microbiology, Federation Meeting, Cold Spring Harbor Laboratory RNA Tumor Virus Meeting, etc.) each year, to the annual review of the Virus Cancer Program, and occasionally to Bethesda for consultation with NCI staff.
- e. Supplies. Includes cost of non-capital equipment, glassware, isotopic and other chemicals, tissue culture glassware, media, and sera, etc. Estimate is based on previous usage of these items.
- f. Shops and Maintenance. These services are provided and charged as required to repair instruments, laboratory facilities, and for fabrication of special items in glassblowing and machine shops. Estimate is based on previous usage of these services.
- g. Glassware Washing. Washing and sterilization of glassware as well as preparation of basic media; costs of labor and materials associated with this are collected here. Estimate is based on previous use of this service (one-third of a labor union person per year).
- h. Utilities. Cost of electricity, heat, air-conditioning, including monitoring and maintenance of these utilities, are charged to each research activity on a square foot basis. Space for this activity is estimated to be 1270 sq. ft. (present rate \$9.00 per sq. ft.).

Technical Proposal

NCI-DOE Cancer Research Program

Retroviral Genetic Expression in Human Cancers:
Analysis by Primer tRNA Binding Approach

Principal Investigator: Wen K. Yang, M.D., Ph.D.
Biology Division, Oak Ridge National Laboratory
Oak Ridge, Tennessee

Proposed Addition to Interagency Agreement:
Department of Energy and
National Cancer Institute

Proposed Period - October 1, 1978 - September 30, 1981

1078806

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1078807

I. INTRODUCTION

This document presents a technical proposal generated by the Viral Oncology component of the Molecular Carcinogenesis Unit at the Biology Division of the Oak Ridge National Laboratory. Proposed for support through the interagency agreement between the National Cancer Institute and the Department of Energy are research projects employing a unique experimental approach to investigate genetic changes in human cancer cells. Recent advances in animal model studies by many investigators clearly indicate an intimate, if not inseparable, relationship between oncogenic information of RNA tumor viruses and specific genomic components of the cells. Also, it is known that these viruses may carry genes for carcinoma induction--an important finding relevant to human cancers. Neoplastic transformation of the cell by RNA tumor viruses generally involves partial (oncogene) rather than total (virogene) genetic expression. For the past several years, the cancer research team at the Oak Ridge National Laboratory has studied various aspects of genetic interactions between host cells and RNA tumor viruses with particular emphasis on cancer prevention. One particular aspect is the phenomenon of host cell tRNA molecules being utilized as primers for the initiation of viral genomic RNA-directed DNA synthesis (reverse transcription) in the life cycle of these tumor viruses. Our effort, in this aspect of study, has resulted in a unique experimental methodology for detection of specific oncogenic expression in the cell (messenger RNA molecules containing "leader" sequences with primer tRNA binding properties). This methodology would be particularly useful for the study of human cancers, where RNA tumor virus involvement might be limited to oncogene expression only.

1078808

A. SCOPE AND OBJECTIVES

The ultimate impact of an environmental carcinogenic agent (be it chemical, radiation, or virus) is an altered expression and/or composition of specific genomes which determine the neoplastic phenotype. In the study of carcinogen-cell genome interaction in animal model systems, investigators working on RNA tumor viruses have the advantage that recently advanced concepts and techniques of molecular biology can be readily employed and thus have achieved rapid progress. However, such an approach to the problem of human cancers appears to be difficult because of a lack of authentic human RNA tumor virus isolates. Research attempts to isolate human RNA tumor viruses have been unsuccessful; this is presumably due to the fact that human beings cannot be genetically manipulated and experimentally controlled like laboratory animals. Also, human cells presumably have a strong repression mechanism to prevent complete RNA tumor virus expression. Further, transforming RNA tumor viruses are usually replication-defective. As clearly revealed in animal model systems, neoplastic transformation of the cell by RNA tumor viruses only involves the oncogenic portion, rather than the whole, of the integrated viral genome. Thus, it is important to consider alternative experimental approaches, other than virus isolation, aiming specifically at the "oncogenes" and/or the defective transforming "virogenes" present in human cancer cells.

The particular experimental approach, which forms the basis of the present research proposal, is to use the property of 'primer-tRNA' binding as a marker to identify the presumed virus-related specific RNA molecules in human cancer cells. It is now well established that reverse transcription of viral genome is initiated with the incorporation of the first deoxyribonucleotide onto a primer which is a tRNA molecule of cellular origin. The primer tRNA is bound to the viral RNA genome by base-pairing of 15-20 nucleotides at the initiation site. All RNA tumor viruses examined have been found to contain such primer-tRNA binding sequences in their genomes. This characteristic sequence property of the viral gene is apparently essential for the survival of RNA tumor virus, since without primer binding no reverse transcription, nor subsequent steps of virus life cycle may take place. Also, RNA tumor viruses

of different phylogenetic origin appear to utilize different primer-tRNA molecules, thus providing a useful marker for the genetic identification of these viruses. Furthermore, in view of the recently discovered "gene-splicing" mechanism of messenger RNA processing, it is possible that messenger RNA representing the oncogenic portion of virogene may contain a spliced "leader" sequence with primer tRNA binding property.

Therefore, the primary purpose of the present proposed research is to examine various human cancers for the presence of RNA molecules which carry the tRNA binding nucleotide sequences. Particular emphasis will be placed on cellular poly(A) RNAs of human cancers, such as breast cancers and leukemias, which have been suggestive of RNA tumor virus association. The specificity of the tRNA binding sequences can be determined by using non-cancer human cells as the control and also by performing reverse transcription reaction to determine the primer activity of the bound tRNA. Secondly, with the particular human cancers in which specific viral primer-tRNA binding sequences are detected, attempts will be made to isolate the cellular RNA species carrying such sequences. Further characterization of the isolated RNA molecules will include assay of messenger RNA activity by cell-free protein synthesis and preparation of copy DNA for clinical studies.

B. BACKGROUND

Briefly described and discussed in the following are five sections of background information related to the theme of the present research proposal.

a. Direct RNA tumor virus studies in human cancers

For the past decade, available virological methods have been extensively employed by many laboratories around the world in an attempt to isolate RNA tumor virus from human cancers. Among the early well-noted virus isolates were the ESP-1 virus, which was presumably of murine origin, and the RD-114 virus (1), which has turned out to be an endogenous virus of the domestic cat (2). While subsequent findings about some of these isolates (e.g., RD-114) are of considerable scientific significance, the fact that they are unrelated to human cancers has caused great disappointment. Recent reports of virus isolation from human leukemia and lymphoma cell cultures (3-5) and non-cancer cell cultures (6,7) have been received generally with caution; it is expected that these investigators have yet to characterize unequivocally the genetic identity of their virus isolates and also to provide strong evidence relating these viruses to human cancers. Thus, up to now no single isolate has been recognized as an authentic human RNA tumor virus by most of the concerned scientists. This indicates that there exists in human cells a strong genetic mechanism to prevent the complete expression of RNA tumor viruses. This also implies that neoplastic transformation of human cells--like that of many animal cells--may involve only the expression and/or occurrence of "oncogenic" gene portion, and not necessarily complete genome, of an RNA tumor virus.

Considerable effort has been directed towards the search for gene products, or "footprints", of RNA tumor virus in human cancer cells. Gallo and co-workers reported finding reverse transcriptase-like DNA polymerase activity in human leukemic cells (8,9). This polymerase activity was found to be immunologically related to the reverse transcriptase of Simian leukemia/sarcoma virus isolates (10). An extensive study by Spiegelman and co-workers with an elegant technique (11) has concluded that virus-like polymerase/70S RNA complexes may be detected in tissues of human leukemias, breast cancers and other malignancies (reviewed in 12); that human breast cancers may contain mouse mammary tumor virus-related gene sequences and human leukemia cells may contain Rauscher murine leukemia virus-like gene sequences; and that these gene sequences appear to be of

exogenous origin (13). Independent studies of Gallo and co-workers on human leukemias have reached generally similar conclusions (review in 14).

Although sensitive and specific radio-immunological methods have been available for detecting viral antigens, relatively few reports have been published with positive findings of the virus expression in human cancers. Sherr and Todaro (15) detected a baboon endogenous virus-related p30 antigen, although in low incidence, in some human tumors. Metzgar and collaborators (16) reported that sera of primates immunized against human leukemic cells reacted with the glycoprotein gp70 of Friend murine leukemia virus. Three groups reported the presence of natural antibodies, reactive with protein components of some primate viruses, in normal human subjects (17-19); however, in a similar study Stephenson and Aaronson detected no such human natural antibodies (20). Employing an assay for cell-mediated immunity, Black and collaborators (21) observed reactivity to mouse mammary tumor virus extracts in some human breast cancer patients. All these findings suggest that there may be partial expression of RNA tumor viruses in some human cancers; however, since almost all of these previous studies concerned gene products other than expression of the oncogenic information of RNA tumor viruses, the positive findings were often of a sporadic nature and could not be generally confirmed.

b. Genetic origin and evolution of RNA tumor viruses

Based on results from earlier studies, the oncogene-virogene hypothesis of Huebner and Todaro (22) and the provirus hypothesis of Temin (23) were developed to explain the origin of RNA tumor viruses. Since the publication of these hypotheses, several important experimental findings have been made. First, type "C" viruses can be induced from apparently normal cells. This was first discovered in mouse cells (24,25), then elegantly demonstrated in baboon placenta (26), and has been actively pursued in various primate culture cells (e.g., 27,28) presumably including human cells. Successful virus isolation is evidently dependent on appropriate gene perturbation for induction and sophisticated means of virus propagation. Second, determination by molecular hybridization has shown that DNA copies of RNA tumor virus may be present in cellular DNA of both normal and tumor cells of the

animal (endogenous), or may be present only in cellular DNA of virus-infected and transformed cells (exogenous). Examples are murine RNA tumor virus isolates (29) for the former and feline leukemia virus and simian leukemia/sarcoma viruses for the latter (30). Third, trans-species transmission of RNA tumor viruses may occur in the evolution of animal species. The ingenious ideas and experimental evidence for this evolution concept have been contributed solely by Todaro and his co-workers (reviewed in 31). Thus, an RNA tumor virus (either endogenous or exogenous) may have its origin from an evolutionally distant species of animal. Fourth, genetic recombination of different viral genes may frequently occur (32) and the oncogenic property may thus be generated. This has been suggested by Hartley and collaborators (32a) in the study of AKR mouse leukemogenesis and also by Scolnick and co-workers (33) in the study of Kirsten and Harvey sarcoma viruses. Therefore, an RNA tumor virus may have a recombinant type of genome with components of different genetic backgrounds. All of these findings have revealed that genetic origin and evolution of RNA tumor viruses are closely associated with, if not inseparable from, phylogenetic evolution of cell genes.

On the basis of these recent findings, it may be expected that an authentic human endogenous RNA tumor virus will eventually be isolated. However, it is also expected that RNA tumor viruses related to human cancers would be very heterogenous and complex in terms of phylogenetic origin and genetic composition. In other words, to study the presumed "oncogenes" of human cancers by the experimental approach of virus isolation, one would have to set a goal for multiple species rather than a single species of human RNA tumor viruses.

c. Oncogenes and replication-defective oncogenic RNA tumor viruses
Many genetic analyses (34-37) and an extensive molecular and genetic study by Duesberg, Vogt, Wang and their co-workers (38) have established the fact that the neoplastic transforming function of avian sarcoma viruses is dependent on the presence of a specific gene sequence, termed the "src" gene, in the viral genome. Recently, Erickson and his co-workers (39) have elegantly demonstrated that the "src" gene is expressed as a putative transforming protein of 60,000 dalton size, which is found in the virus-transformed cells but not in the virion; two laboratories

(40,41) subsequently reported data confirming this finding. This is important because in this case oncogenic function can now be defined in terms of specific molecules and oncogenes may thus represent physical gene entities in addition to functional meanings. Furthermore, an examination of the literature (42) clearly indicates the existence of avian RNA tumor viruses, which cause carcinomas and leukemias in chicken; these carcinomas-causing and leukemia-causing viruses are very different from the sarcoma-causing Rous virus in terms of viral genome composition (42b). (In mammalian systems, mammary tumor viruses, leukemia viruses and sarcoma viruses, respectively, associated with different neoplasms are recognized to be distinct genetic entities.) Thus, the genes responsible for neoplastic transformations, termed "onc" by Duesberg and Baltimore (43), can be subclassified into "src", "carc", and "leuk" genes. This implies that RNA tumor viruses may be related also to cancers of epithelial cell origin (carcinomas), which constitute the majority of human malignant diseases.

Investigators working with mammalian sarcoma-causing RNA tumor viruses have long noticed that these viruses are generally replication-defective and hence require helper viruses for propagation in cells (e.g. 44-47). Upon infection with sarcoma viruses alone, the cell is neoplastically transformed but does not subsequently produce infectious sarcoma virus progeny. The sarcoma virus genome in these non-producer cells, or S^+L^- cells (48) can be rescued by additional infection with "helper" virus. The replication-defectiveness was also found in the avian carcinoma-causing MH2 virus, as well as the avian leukemia-causing MC29 virus (42), and therefore could be a common phenomenon among RNA tumor viruses that carry on "onc" gene. And yet, these replication-defective "onc" gene carrying RNA tumor viruses are generally the result of laboratory research which represents an extensive artificial selection. Such results have been obtained by Rapp and Todaro (49), who made long serial passages of apparently non-oncogenic and weakly oncogenic murine type C viruses in mouse cancer cells and observed successively increasing oncogenic potentials in these viruses. Thus, "onc" genes might exist in nature (or in human cancer cells) as self-sufficient genetic units with rare probability

of evolving into the replication-defective RNA tumor viruses, which in turn rarely evolve into a helper-independent RNA tumor virus.

Another important finding pertains to the biological nature of the "src" gene sequences. Working on the genome structure of Kirsten and Harvey sarcoma viruses, Scolnick and co-workers (50,51,33) have discovered that these viruses contain in their genomes some gene sequences derived from rat genetic information and that the oncogenic potential of these viruses are apparently associated with, if not derived from, the acquisition of the rat gene sequences. The presumed "src" sequences are expressed in rat cells as RNA molecules having characteristic properties of an endogenous RNA tumor virus (52,53), and appear to be well-conserved in the evolution of rodent species (54). Working on the avian sarcoma viruses, and employing specific cDNA from "src" gene sequence, Staehlin, Spector, Varmus, Bishop and co-workers obtained evidence that this gene, named "src", is present in the chicken cell DNA genome (55) and appears to be expressed to the same extent in normal and chicken tumor cells (56,57); this apparently paradoxical finding might be due to the particular methodology used by these investigators for the preparation of cDNA probes. Thus, the results of all research teams have agreed on one point, namely, that the "src" gene sequences, defined by molecular hybridization, are normal cell gene components. The results of Scolnick and co-workers further imply that the normal cell gene sequences have undergone some rather subtle modification, presumably toward retrovirus form, to gain the oncogenic property. The nature of the gene modification remains to be elucidated.

d. Gene splicing and the "leader" sequences in RNA tumor virus-specific messenger RNAs

The phenomenon of gene splicing was first discovered in the transcription products of adenovirus in infected cells (58-59), and has now been established to be commonly taking place during gene expression, such as in the synthesis and formation of hemoglobin mRNA, immunoglobulin mRNA, ribosomal RNA, and even transfer RNA (reviewed in 58). In all of these mRNA cases, the DNA coding sequences which will ultimately be translated into amino acid sequences of a protein product are not continuous as a whole but generally consist of two parts interrupted by some insertion DNA sequence.

The phenomenon of gene splicing is important for RNA tumor viruses mainly in two ways. First, the genomic RNA of 30-35S size found within the virion of RNA tumor viruses is, structurally speaking, a messenger RNA with a poly(A) sequence at 3' end and capping at 5' end. Second, virus-specific mRNAs found in the cell are of different sizes representing gag/pol/env/onc, env/onc, and onc genes of the virus, such as in the study of Rous sarcoma virus-infected cells (60). The exact structure of these virus-specific mRNAs is still unknown. Recent evidence has indicated that gene splicing may occur during the processing of these mRNAs. Weiss, Varmus and Bishop (61) reported that cDNA to the 5' end of Rous sarcoma virus genomic RNA hybridized with a presumed env-containing mRNA isolated from the infected cells. Mellon and Duesberg (62) reported that finding that "sub-genomic, cellular Rous sarcoma virus RNAs contain oligonucleotides from the 3' half and the 5' terminus of virion RNA." Working on murine leukemia virus by the technique of heteroduplex mapping with long copy DNA, Rothenberg, Donohue and Baltimore (63) observed that at the 5' end of the 21S glycoprotein mRNA from virus-infected cells there was a stretch of 500-600 nucleotides which were mapped at the 5' end of the viral genome RNA. The "leader" sequence of 500-600 nucleotides may thus be spliced onto the 5' side of structure gene proper of the env during the processing of this virus-specific mRNA. It is thus very important to determine whether or not the 5' "leader" sequence is present in the mRNA molecules which contain the "onc"-specific information and, furthermore, whether or not this kind of gene structure organization is responsible for the generation of neoplastic function in a normal cell gene component.

e. Primer tRNAs

Possible involvement of cellular tRNAs in genetic functions of RNA tumor viruses was first implied by the finding of Erikson and Erikson (64) that 4S RNA molecules, with sequence characteristics of tRNAs, are constituents of the 70S RNA complex within virion particles. In a study of reverse transcription, Verma and co-workers (65) observed that the first deoxyribonucleotide incorporated was covalently linked to a priming RNA of small size. Canaani and Duesberg (66) subsequently reported that the primer is a unique 4S RNA molecule which dissociated from viral genomic template RNA with a distinct melting temperature. Bishop and co-workers

suspected that the primer was a tRNA, but it was by Dahlberg and co-workers' collaboration with them that tRNA^{trp} was revealed to be the primer in Rous sarcoma virus (67).

Employing a very different experimental approach, Waters, Yang and co-workers demonstrated that tRNA^{trp} also served as the primer for reverse transcription in avian myeloblastosis virus (68). In an in vitro hybridization reaction including viral genomic RNA and total cellular tRNA mixture, only selective tRNA species became associated with the viral RNA (69); this implies a stretch of complementary sequence between viral genomic RNA and the primer tRNA. This is now known to involve 17 base sequences of the primer tRNA structure from 3' CCA end to the T4CG loop (70). The site of primer tRNA binding was found to be at the 5' end side of the viral genomic RNA (71). Precise measurement by the size of copy DNA product from "strong-stop" reverse transcription indicates that it is approximately 200 bases away from the 5' terminus (72).

From the above-mentioned results, two points are particularly important: First, RNA tumor viruses generally initiate the reverse transcription of the RNA genome by the utilization of a unique host cell RNA molecule, which normally serves to recognize a specific genetic code and mediates the incorporation of amino acid into protein. Second, and more significantly, the primer tRNA binding sequence is evidently included in the "leader" sequence at the 5' end of virus-specified messenger RNA molecules.

C. CURRENT RELATED RESULTS OF PRIMER tRNA STUDIES IN THIS LABORATORY

For more than a decade up to 1972, the Macromolecular Separation Program of the Biology Division of Oak Ridge National Laboratory was well noted for its technological development in the separation of transfer RNAs. The various reversed-phase chromatographic systems developed here have been applied widely (73). Through the coordinative effort of G. David Novelli, our laboratory took advantage of this development and did studies on iso-accepting tRNAs in mammalian tumor cells and tissues (e.g. 74-78). From these studies useful techniques were also devised for characterization of tRNAs in mammalian systems (79). Our recent investigation into the problem of primer tRNAs of RNA tumor viruses has thus utilized the methodology based on our past research experience on eukaryotic tRNAs. We have

encountered various technical difficulties, which are unique to the RNA tumor viruses, and attempts to solve these difficulties have resulted in technical and conceptual modifications of the investigations. These results are very briefly described and discussed here.

(i) Candidate primer tRNAs of various RNA tumor viruses

In this laboratory we determine the identity of primer tRNA for a RNA tumor virus on the basis of three criteria -- namely, that it is the last tRNA species to be heat-dissociated from viral 70S RNA complex, that the tRNA can selectively form duplex with viral 30-35S genomic RNA in an in vitro hybridization reaction, and that after forming duplex with it the viral genomic RNA regains template activity with reverse transcriptase. This is different from an approach taken by Dahlberg and others, who determine the identity of primer tRNA by brief reverse transcription using [32 P]-labeled virus preparations, isolate the primer with short nascent DNA pieces, and perform sequences analyses. Whereas these investigators obtained definite answers to the sequence property of the primer, our experimental approach more readily identified the amino acid accepting property of the tRNAs. In our studies all three criteria of a primer tRNA have been fulfilled by tRNA^{trp} in avian myeloblastosis virus (68) and also by tRNA^{pro} in AKR and Rauscher murine leukemia viruses (80). As an example, Table 1 shows the results of differential thermo-dissociation of tRNAs from genomic RNA of AKR murine leukemia virus, revealing that tRNA^{pro} is the "candidate" primer tRNA of this virus. Our subsequent experiments of selective duplex formation and reverse transcription as well as results from other laboratories (81) have established the authenticity of tRNA^{pro} as the primer tRNA of murine leukemia viruses. These results have also indicated that the differential thermo-dissociation experiment is rather dependable for determination of the primer tRNA candidate. Table 2 lists the identity of candidate primer tRNAs of 15 different RNA tumor viruses, determined mainly by differential thermo-dissociation of the viral 70S RNA. These viruses appear to form three classes according to the candidate primer tRNA identified: tRNA^{trp} (avian viruses); tRNA^{pro} (murine viruses, woolly monkey virus and feline leukemia viruses) and tRNA^{gly} (RD114 cat virus and baboon M7 virus). We are at present studying avian reticuloendotheliosis virus (in collabora-

TABLE 1. COMPARISON OF THE SPECIFIC AMINO ACID IRNAs IN AKR MOUSE EMBRYO CELLS AND IN AKR MURINE LEUKEMIA VIRUS 4S RNAs.

Source of RNA	RNA (μ g)	Total radioactivity ^a	Percentage of total radioactivity identified as:																
			Trp	Lys	His	Arg	Asp	Ser	Glu	Pro	Gly	Ala	Val	Met	Ile	Leu	Tyr	Phe	
Cellular 4S RNAs																			
AKR mouse embryo cells (1) ^b	35	73,120	2.3	11.8	3.3	18.2	3.6	17.4	2.2	3.0	2.4	2.5	14.6	1.8 ^c	3.3	9.4	1.7	2.5	
AKR mouse embryo cells (2)	16	48,070	2.3	12.5	2.9	17.5	2.8	17.2	1.4	2.5	2.4	1.1	13.2	7.3	2.7	9.8	1.8	2.4	
Viral 4S RNAs																			
"Free" 4S RNA	28	43,210	1.1	22.0	11.1	24.3	2.5	5.1	1.2	13.3	8.2	2.2	3.0	0.5 ^c	3.1	1.4	0.5	0.5	
"70S-associated" 4S RNA																			
High salt 60°	d	10,390	6.9	20.0	3.0	37.6	1.7	7.8	8.2	2.8	2.1	1.0	0.6	0.3 ^c	4.0	3.8	0.1	0.2	
High salt 60-80°	d	1,350	4.4	5.9	ND	8.5	0.9	3.6	6.6	59.8	1.4	5.0	ND	ND ^c	1.0	3.0	ND	ND	
Low salt 60° (1)	d	9,700	7.4	19.9	2.4	34.6	2.0	8.1	9.1	2.7	2.3	0.7	1.6	0.4 ^c	4.2	4.1	0.2	0.2	
Low salt 60° (2) ^e	d	8,000	7.5	21.2	1.4	39.3	1.6	9.6	4.6	1.6	2.0	0.7	0.7	0.9	4.0	4.9	ND	ND	
Low salt 60-80° (1)	d	890	3.8	6.5	1.3	10.8	ND	6.4	6.8	54.5	0.9	3.9	ND	ND ^c	2.0	3.0	ND	ND	
Low salt 60-80° (2) ^e	d	720	4.4	8.4	ND	12.0	1.3	8.0	5.5	49.6	1.7	2.6	ND	0.9	2.2	3.4	ND	ND	

^aSum of radioactivities eluted from the amino acid analyzer in peak positions corresponding to the indicated amino acids.

^bNumbers in parentheses are to indicate different RNA samples.

^cNon-maturing concentrations of methionine in aminoacylation reaction.

^dThe high salt 60° and 60-80° samples and the low salt 60° and 60-80° samples (1) were derived from equivalent portions of 70S RNA (110 μ g each); the low salt samples (2) were derived from 90 μ g 70S RNA.

^eThese samples (2) were prepared from a different virus preparation than were samples (1).

ND = none detected.

TABLE 2. PRIMER tRNA DETERMINATION

Retrovirus	Step 1	Step 2	Step 3
Avian myeloblastosis virus	trp	trp	trp
Rous Sarcoma Virus	trp	trp	trp
AKR-MuLV	pro	pro	pro
Moloney-MuLV	pro	---	---
Rauscher-MuLV	pro	pro	pro
Gross N-tropic MuLV	pro	---	---
WN 1802 B-tropic MuLV	pro	---	---
Balb/c Amphotropic MuLV (CCL64)	pro	---	---
FeLV-Rickard	pro	---	---
FeLV-Theilen	pro	---	---
SiSv-Wooley monkey	pro	pro	---
RD 114 (TE 385)	gly, asp.	---	---
RD 114 (CCL 64)	gly, asp.	---	---
BaV (M ₇ A204)	gly, asp. (pro)	---	---

- Step 1. Heat denaturation of viral 60-70S RNA at various temperatures in a stepwise fashion and identification of the amino acid accepting properties of the released tRNA fractions (the most tenaciously bound tRNA species is the candidate primer)
- Step 2. Purification of the tRNA species and demonstration that it can hybridize with viral 30-40S RNA
- Step 3. In vitro reaction using an isolated reverse transcriptase preparation to demonstrate that the 30-40S RNA regains its template activity after hybridization with the purified tRNA species.

tion with C. Y. Kang) in view of the fact that these viruses appear to have considerable sequence homology with mammalian RNA tumor viruses at the 5' end of the genome. The data collected thus far are still incomplete; however, they have clearly indicated that RNA tumor viruses of different phylogenetic origin may utilize different cellular tRNA species as the primer for reverse transcription. This implies that primer tRNA binding sequences may differ according to genetic and evolutionary characteristics of the RNA tumor viruses.

(ii) Possible cellular origin of primer tRNA binding sequences

One of the experimental steps for primer tRNA characterization in this laboratory has been to incubate viral genomic RNA with total tRNA mixtures in an in vitro hybridization reaction to demonstrate specific duplex formation (69). In performing control studies for this experimental step, we made an unexpected finding which has become significant (84). Mouse cell 28S non-poly(A) RNA showed hybridization of tRNA radioactivity in the hybridization reaction (Fig. 1). RPC-5 chromatography revealed that selective tRNA species were involved in the hybridization (Fig. 2). The bound tRNA were incubated in an aminoacylation reaction and the amino acids accepted by the tRNA were then identified by an amino acid analyzer; as shown in Fig. 3B, mouse cellular 28S RNA binds predominantly tRNA^{pro} which also serves as the primer for all murine leukemia viruses. 28S Non-poly(A) was prepared by phenol extraction, oligo-dT cellulose chromatography and sucrose gradient sedimentation; such preparations from livers and embryos of different mouse strains all showed the same selective binding of tRNA^{pro}. Also, similar results were obtained with 28S RNA prepared from isolated 60S ribosome subunits. Even with large excesses of tRNAs included in the hybridization, only 0.1 to 0.5 molecules of tRNA^{pro} was bound per molecule of 28S RNA. Thus, although it is likely that selective tRNA^{pro} binding is due to a subpopulation of 28S ribosomal RNA, the possibility that it is due to some RNA species contaminating the 28S rRNA preparation has not been excluded. Mouse 18S ribosomal RNA appears to selectively bind different tRNA species like tRNA^{glu}, tRNA^{lys} and tRNA^{gly} (Fig. 3D). Melting temperature of 28S RNA·tRNA^{pro} duplex (Fig. 4) is very similar to murine leukemia

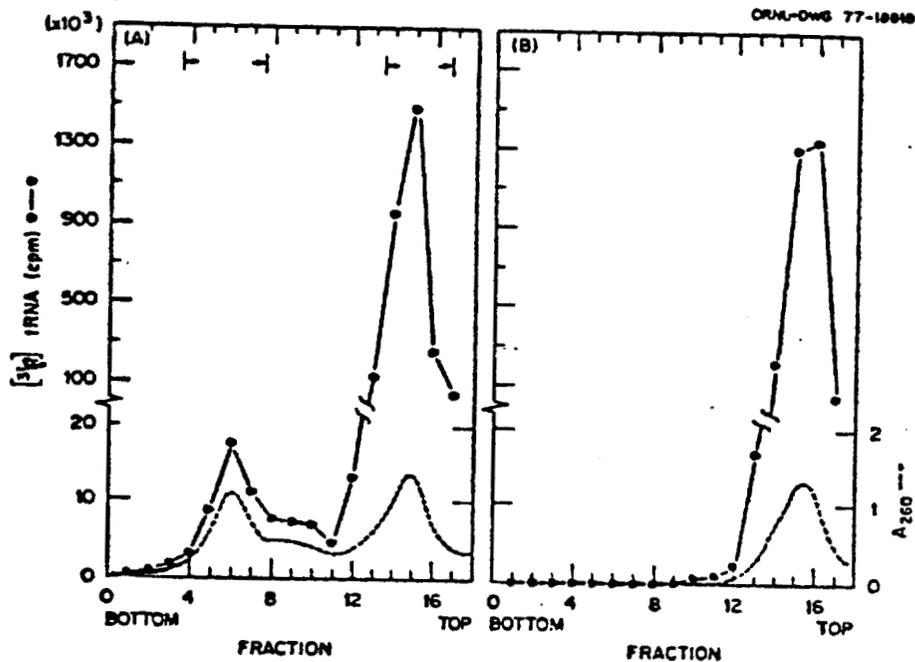


Figure 1. Sucrose gradient sedimentation of a mixture of 28S RNA and $[^{32}\text{P}]\text{tRNA}$ (A panel) and $[^{32}\text{P}]\text{tRNA}$ alone (B panel) after in vitro hybridization reaction. The reaction mixture (0.2 ml) of 4.8 A_{260} units of 28S RNA and 5.0 A_{260} units of $[^{32}\text{P}]\text{tRNA}$ (both from NIH 3T3 cells) was layered onto a 3.8-ml 10–30% sucrose linear gradient containing 1.0 mM TrisCl (pH 7.6), 10 mM NaCl, 1 mM EDTA, and 0.05% sodium dodecyl sulfate. After centrifugation in a Spinco SW 56 rotor at 50,000 rpm for 4.5 hr, 0.2 ml fractions were collected from the bottom of the gradient. The fractions were diluted to 1 ml for determination of absorbance at 260 nm and of radioactivity by Cerenkov counting.

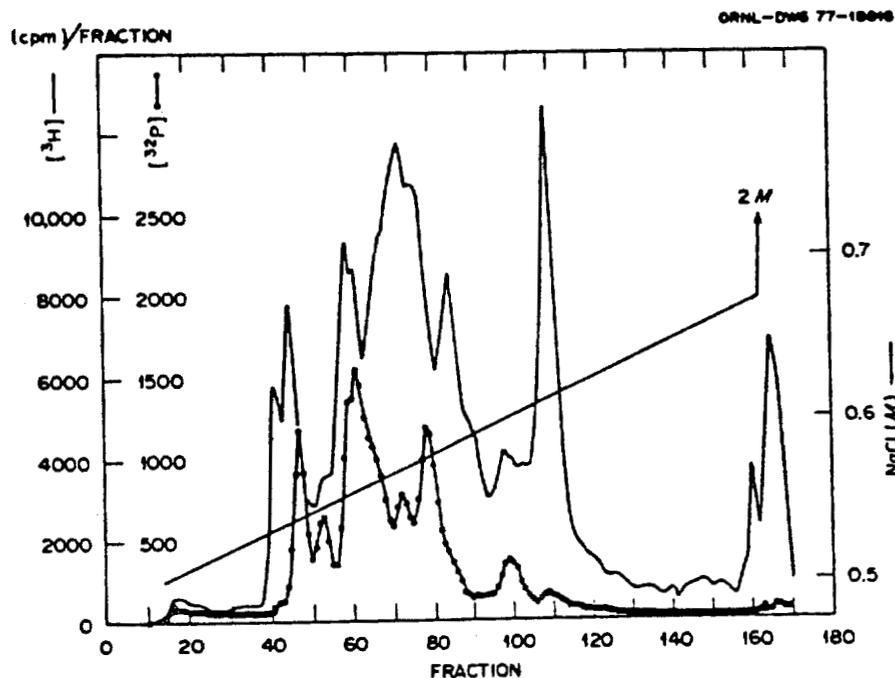


Figure 2. Reversed-phase chromatography of the 28S-associated 4S RNA (^{32}P) and the total tRNA (^3H) preparations derived from NIH 3T3 cells. The sample containing approximately 10 μg of tRNA was applied to a 0.6 X 30-cm RPC-5 column and eluted at 20°C with 200 ml of 0.5–0.7 M NaCl linear gradient containing 10 mM Na-acetate (pH 4.5), 10 mM MgCl_2 , 1 mM EDTA, and 2 mM 2-mercaptoethanol. Acid precipitable radioactivities of 1-ml fractions were measured (8).

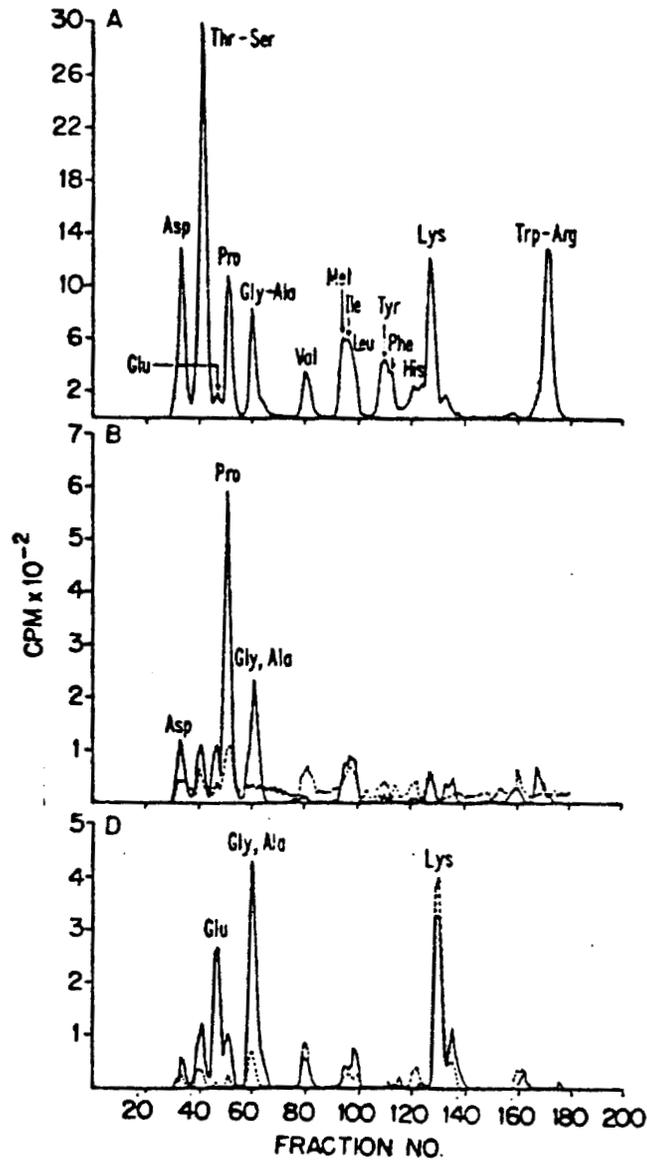


Figure 3. Profiles of ^3H -labeled amino acids representing amounts of individual tRNAs in total tRNA of BALB/c mouse liver (A), in pool a gradient fraction of 28S rRNA-tRNA complexes (B), in pool a fraction of 18S rRNA-tRNA complexes (D). The RNA fractions were either heat-dissociated (solid line) or without heat dissociation (dotted line) and aminoacylated with 17 ^3H -labeled amino acids. Amino acids accepted by tRNAs were subsequently discharged and chromatographed in an amino acid analyzer column.

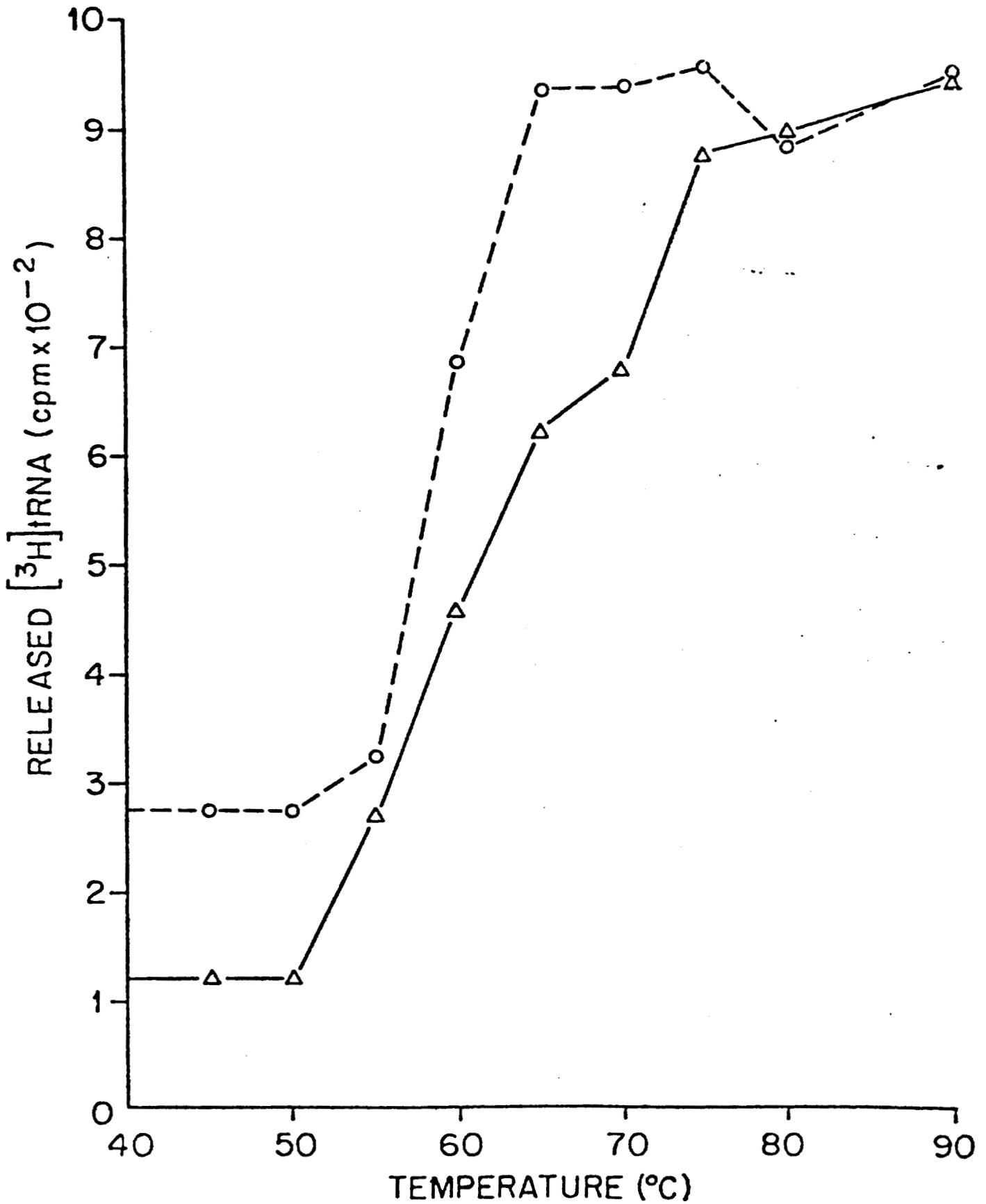


Figure 4. $[^3\text{H}]$ uridine-labeled tRNA released from 28S rRNA-tRNA complex (●) and 18S rRNA-tRNA complex (△) at various temperatures in 0.1 M NaCl, 0.01 M TrisCl (pH 7.6), and 0.001 M EDTA.

virus genomic RNA·tRNA^{Pro} duplex which is known to involve a complementary sequence of 17 nucleotides. The exact structure of binding sequences of mouse cell 28S RNA·tRNA^{Pro} is being investigated (in collaboration with J. E. Dahlberg).

We have extended the study to the liver 18S and 28S RNAs of various vertebrate species (Table 3). Although still preliminary in nature, the results revealed a few points of interest. First, the RNA preparation from each animal species consistently showed selective tRNA binding. Second, the predominant tRNA species bound by the 28S RNA of an animal species seem to correspond to the primer tRNA of endogenous RNA tumor viruses of this animal species (67,68,80,81,84). Thus, tRNA^{trp} is specifically bound by chicken cell 28S RNA, tRNA^{Pro} by mouse cell tRNA, and tRNA^{Gly} by baboon cell 28S RNA. Third, the selective binding of tRNA seems to vary in animal species according to phylogenetic evolution. In this aspect, fish, amphibian, reptiles, birds, and mammals seem to form distinct tRNA binding patterns.

Definitely much more work is needed to determine whether or not these preliminary results of tRNA binding by cellular RNA represent molecular origin of primer tRNA binding sequences found in the RNA tumor virus genomes and, thus, whether or not these findings can be employed to predict the primer tRNA of endogenous RNA tumor viruses of an animal. This would be of considerable practical value especially in the study of human RNA tumor viruses.

(iii) Primer tRNA binding of poly(A) RNA from cells infected with or transformed by RNA tumor viruses

Identification of candidate primer tRNA of an RNA tumor virus by the differential thermodissociation experiment generally requires 50 µg or more of intact 70S RNA sample, which is often difficult to obtain with poorly reproducing RNA tumor viruses. Our attention was hence drawn toward intracellular virus-specific RNAs, which have been reported to be present in considerable quantity in virus-infected and/or-transformed cells (e.g. 84,85). It would be interesting to see if these intracellular virus-specific RNAs are similar to virion 70S RNA in containing the primer tRNA. Mouse cells infected with murine leukemia virus were used for prepa-

TABLE 3 - PREDOMINANT tRNAs BOUND TO 18S AND 28S RNAs IN IN VITRO
HYBRIDIZATION REACTION

Animal Species	AMINO ACID ACCEPTANCE OF tRNA BOUND TO	
	18S RNA	28S RNA
Fish (trout)	Asp	Asp
Amphibian (frog)	Gly	(Asp) ^a
Reptile (snake)	Asp	(Phe, Trp)
Bird (chicken)	Trp	<u>Trp</u> ^b
Mammals		
Mouse	Gly, Glu	<u>Pro</u> ^b
Rat	(Gly, Glu)	(Pro, Gly)
Chinese hamster	(Gly)	(Gly)
Rabbit	Gly	Gly
Cat	(Gly)	<u>(Gly)</u> ^b , Ser)
Dog	Gly	Ser, Leu
Pig	(Ser)	(Ser)
Baboon	(Gly)	<u>(Gly)</u> ^b , Ser)
Rhesus monkey	(Gly)	(Ser)
Human (A ₂₀₄)	Glu	Glu
(HT1080)	(Gly)	(Ser, Gly)

^a Results from one experiment performed and needed to be confirmed.

^b Apparent correspondence to the primer tRNA of endogenous RNA tumor viruses of the animal species.

TABLE 4.

Proline tRNA Released by Thermodissociation
From MuLV RNA and Cellular RNAs

<u>Sources of "melted-out" 4S RNA</u>	<u>Proline Accepting Activity</u> <u>pmoles/A₂₆₀</u>
AKR MuLV 70S RNA	640
AKR producer fibroblasts	
>28S poly(A)-RNA	<0.5
28S RNA	<0.5
tRNA	43
Balb/c mouse liver	
>28S poly(A)-RNA	<0.5
28S RNA	<0.5
tRNA	29

ration of poly(A) RNA. Since tRNA^{PRO} is known to be the primer of murine leukemia viruses, release of tRNA^{PRO} by heat treatment of the poly(A) RNA preparation would indicate the presence of duplex forms of intracellular virus-specific RNA and primer tRNA. As shown in Table 4, very little or no tRNA^{PRO} was released from a single 35S size poly(A) RNA preparation from the infected cells whereas significant tRNA^{PRO} was released from virion 70S RNA by the same heat treatment. These results would suggest that duplex formation between the primer tRNA^{PRO} and genomic RNA of murine leukemia viruses occurs extracellularly after virus budding.

Next, we performed the step 2 experiment (see Table 2 legend) with poly(A) RNA preparations of various mouse cells, to examine the content of RNA species which were capable of binding the primer tRNA. In this study, poly(A) RNA preparations were respectively incubated with labeled tRNA^{PRO} in an in vitro hybridization reaction. After hybridization, poly(A) RNAs with the bound tRNA^{PRO} were isolated from free tRNA^{PRO} by oligo(dT)-cellulose chromatography. As shown in Table 5, poly(A) RNA isolated from SC-1 cells infected with a B-tropic retrovirus bound considerably more tRNA^{PRO} than poly(A) RNA preparations from non-infected mouse cells. However, it appeared that poly(A) RNA from non-infected SC-1 cells, transformed non-producer K-BALB cells and A₂₀₄ cells infected with M₇ baboon endogenous virus also showed significant binding of tRNA^{PRO}. The size of tRNA^{PRO} binding poly(A) RNAs was obscured by considerable aggregation of poly(A) RNA from hybridization reaction. For this reason, poly(A) RNAs were first separated into different size fractions by sucrose gradient sedimentation. Each fraction was then tested for tRNA^{PRO} binding activity in the in vitro hybridization. Results of such an experiment revealed that tRNA^{PRO}-binding poly(A) RNAs were of at least three apparent sizes (20-22S, 28-30S and 35-38S) in the B-tropic virus infected SC-1 cells, only one size (28-30S) in the non-infected SC-1 cells, and two apparent sizes (28-30S and 33-35S) in the transformed non-producer K-A31 cells.

Although more work is needed to characterize the binding interaction of cellular poly(A) RNAs and the primer tRNA, the above preliminary results demonstrated that there exist in retrovirus-infected and -transformed the cells certain poly(A) RNA molecules which resemble the genomic RNA of RNA tumor viruses in the content of a primer tRNA binding sequence. At present we are

defining the hybridization conditions optimal for poly(A) RNAs and also examining the ability of the poly(A) RNA·tRNA^{Pro} duplexes to serve as substrate for in vitro reverse transcription.

TABLE 5. Binding of tRNA^{Pro} by various cellular poly(A) RNA preparation in in vitro hybridization reaction.

Poly(A) RNA Cell Source	[³ H]tRNA ^{Pro} Bound (cpm)
SC-1 cells infected with WN 1802B virus	16,860
SC-1 cells (not-infected)	5,477
K-MSV transformed K-A31 cells (non-producer)	6,145
BALB/c 3T3 cells, clone A31, not infected	1,930
BALB/c mouse liver	2,222
Human A ₂₀₄ rhabdomyosarcoma cells	1,435
A ₂₀₄ , infected with M ₇ virus	3,620
None (tRNA ^{Pro} alone)	1,716

Ten A₂₆₀ units of poly(A) RNA was incubated with 100,000 cpm (~0.5 µg) of [³H-uridine] labeled tRNA^{Pro} in 0.5 ml of hybridization solution (10 mM Tris Cl pH 7.6, 500 mM NaCl, 1 mM EDTA, and 0.1% SDS) at 60°C for 18 hrs. After hybridization, the incubation mixtures were directly applied to oligo(dT)-cellulose column. The [³H]tRNA^{Pro} bound and subsequently eluted by the buffer solution containing 10 mM NaCl from the column was determined.

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II. TECHNICAL PROPOSAL

A. Rationale for the Proposed Research

Generation of the present research proposal is based on our consideration of three particularly pertinent questions: Why RNA tumor virus research is important for human cancer problems? Why the experimental approach of primer tRNA analysis is especially useful for this aspect of RNA tumor virus research? And, whether or not the technical development of this laboratory for the primer tRNA work is sufficient to obtain unambiguous and useful answers.

As described in the Background section, recent research has established three important points about RNA tumor viruses: (i) In nature these viruses are generally endogenous and stay associated with the cell genome. This indicates that the classical concepts of infectious diseases cannot be applied to RNA tumor viruses and thus epidemiological data of human cancers revealing no horizontal transmissible etiological agents is not necessarily incompatible with the idea of RNA tumor viruses being important to human cancers. (ii) Almost all RNA tumor viruses capable of causing neoplastic transformation of the cell are "defective"; this explains the difficulty of isolating an authentic human RNA tumor virus with both infectious and transforming potentials. (iii) These viruses are genetically heterogenous on the oncogene component (src, carc, leuk) and also in other gene components. This suggests that RNA tumor viruses may be important for carcinomas as well as for sarcomas. From these experimental findings, a unique and novel concept of onco-pathogenesis has evolved. This concept implies that endogenous RNA tumor viruses are usually under strict genetic control in the cell and that the neoplastic transforming property is expressed either by a change in viral gene constitution (e.g. genetic recombination) or by impaired cellular control mechanism. This is important since from evolutionary point of view the basic genetic mechanism would be similar in man as in experimental animals, and also since the genes and unique gene expression of endogenous RNA tumor viruses could be the specific targets for chemical carcinogens as well as cancer-causing physical insults. However, before this concept is applied to human cancers, it is necessary to determine whether or not endogenous RNA tumor virus are

present in normal human cells, and also whether or not altered expressions of these endogenous virus occur in human cancer cells.

The most direct approach to the problem of human endogenous RNA tumor viruses is of course by induction, isolation and in vitro propagation of these viruses. However, such approach has had obvious technical difficulties, as clearly revealed by many previous works. To circumvent these difficulties, one alternative approach is to look for viral genomic RNAs intracellularly. In this regard, the basic criterion for recognizing the viral genomic RNAs would be their capacity to be reversely transcribed. Since reverse transcription is initiated from a primer tRNA, this means that the intracellular viral genomic RNA molecules should be capable of binding primer tRNA. There are mainly three advantages for adopting the primer tRNA approach to the study of human cancers. First, the heterogeneity and evolutionary relationship of endogenous RNA tumor viruses may be assessed by both qualitative and quantitative measurement of the bound primer tRNAs, the multiplicity of which (including isoaccepting tRNAs) has been well characterized and hence distinguishable. Second, an RNA molecule with a bound primer tRNA can be reversely transcribed to obtain copy DNA product for further structural and molecular hybridization analysis. Third, the complementary nucleotide sequence responsible for the binding of primer tRNA may provide a means for the isolation of viral genomic RNAs from other cellular RNA molecules. Since it is now known that the "leader" sequence, which includes the primer tRNA binding site, is spliced onto the subgenomic sizes virus-specific messenger RNAs, the primer tRNA approach may thus detect subgenomic as well as the genomic RNA of RNA tumor viruses. In other words, this experimental approach may detect not only complete but also partial expression of retroviruses in the cell.

In taking the experimental approach of primer tRNA binding to study human cancer cells, there are two major technical considerations. One relates to the sensitivity of the detection. It is likely that the endogenous retrovirus-specific RNA molecules may be present only in a few copies per cell, thus requiring a highly sensitive method for their detection. With presently available labeled amino acids of high specific

activity for assaying tRNA by aminoacylation and [γ -³²P]ATP for 5' end labeling of tRNA, the required high sensitivity of detection has become possible. Also, the copy number of intracellular endogenous retrovirus-specific RNA molecules may be stimulated to increase under certain cell growth conditions and after virological and chemical treatments of the cell. The other major technical consideration is the specificity of primer tRNA binding. It is now known that endogenous retroviruses of different phylogenetic origin may utilize different tRNAs as the primer. Therefore, human endogenous retrovirus-specific RNA may bind a tRNA species other than tRNA^{trp} or tRNA^{pro}. In this regard, our recent study on the possible cellular origin of primer tRNA binding nucleotide sequence would favor the consideration of tRNA^{gly} and tRNA^{glu} as possible candidates of primer tRNA for human endogenous retrovirus and, further, provide a basis for tracing the phylogenetic origin of the possible exogenous retroviruses in human cancers. Recently, one of the most puzzling facts noted in viral carcinogenesis is that the genetic sequence (src) responsible for cell neoplastic transformation is found in comparable levels in normal cells as in transformed cells (by molecular hybridization approach). The specificity and the sensitivity of the primer tRNA binding approach would be able to determine whether or not the apparently similar "onc" RNAs of normal cells and of neoplastically transformed cells are different in the "leader" sequences, thus causing different biological effects.

B. SPECIFIC AIMS

a. To isolate poly(A) RNAs from various human normal and cancer cells; to qualitatively and quantitatively compare their capacity to bind selective tRNA species; to assess the primer property of the bound tRNAs by reverse transcription reaction; and to determine whether different types of human cancers contain poly(A) RNA of different tRNA binding specificity.

b. Structure analysis of tRNA·poly(A) RNA binding nucleotide sequences as well as reversely transcribable 5' end sequences of the tRNA-binding site in the poly(A)RNA, by employing nucleotide sequence determination and molecular hybridization.

c. To purify the specific poly(A)RNAs by using the unique "primer tRNA" binding specificity.

d. Characterization of the specific poly(A)RNAs, including messenger RNA activity by protein synthesis assay and possible changes of level during neoplastic process.

C. METHODS OF PROCEDURE

(i) Investigations on selective tRNA binding to poly(A)⁺ RNAs of human cancer cells.

(a) The general strategy for establishing selective and specific binding of tRNAs will include three major kinds of experiments. First, poly(A) RNA is incubated with total mixture of tRNA in an in vitro hybridization reaction; the hybridized poly(A)RNA·tRNA complexes are isolated; and the bound tRNAs are identified by the amino acid accepting activity. Second, the specificity of tRNA binding is ascertained by employing purified tRNA species and poly(A) RNA of defined size and also by determination of the melting temperature of the duplex. Third, the poly(A)RNA·tRNA duplex is incubated in a reverse transcription reaction to examine the priming activity of the bound tRNA. These three kinds of experiments are in principle similar to the experiments we have devised for the determination of primer tRNAs of viral 70S RNA (see Table 2 legend) except that the virion 70S RNAs already contain the primer tRNA molecule whereas poly(A) RNAs of the cell need to be hybridized first to the primer tRNA before subsequent experiments.

(b) Human cell materials:

- Fibrosarcomas (e.g. HT1080, 8387 cells) and normal fibroblasts (e.g. HSBP and WI38 cells)
- Paired cell culture of breast cancer and normal tissues (mainly fibroblasts); established lines of breast cancers (e.g. ALAB cells); and frozen surgical breast cancer specimen
- Cultured line of myelogenous leukemias (e.g. K562); and lymphocytic leukemias (T- and B-lymphocyte types); fresh leukemic cells from leukopheresis; spleen of normal human donor
- Lung cancers, specimen from surgical sources, and established lung cancer cell culture (e.g. A549)
- Other human cancer cultured cells (optional) such as rhabdomyosarcomas (TE31 and A₂₀₄), osteogenic sarcomas (TE85), cervical carcinomas (HeLA) and malignant melanomas (A₈₇₅)
- Hepatocarcinoma and normal liver tissue from surgical sources (optional).

The above six kinds of human cell materials (last two optional) are selected for the present study on the basis of one or more of the following criteria: availability of normal cell counterparts as a control; indication of possible retrovirus involvement by previous studies; high incidence in human population; and possibility of growing in large quantity by cell culture facility and manpower of this laboratory. For the cell and tissue materials, helpful assistance would be expected from the Office of Resources and Logistics of VOP-NCI and the contractors, who have collected various human cancer tissues in frozen state. For human cancer cell lines, the Naval Research Laboratory at Oakland, CA (Drs. Nelson-Rees and Hacket) is a valuable source because of their collection of paired cell cultures from cancerous and normal tissues of same patients (same genetic background) and, more importantly, because of their careful cytogenetic characterization of cell lines. The cell lines with designated names are already collected in this laboratory and can be grown in large quantity either as monolayers or as suspensions. Fresh leukemic cells and cancer tissue materials may also be obtained locally

through the help of oncologists, hematologists, surgeons and pathologists (e.g. at The University of Tennessee-Memorial Research Hospital, Knoxville). If necessary, surgically removed hepatocarcinomas and normal human liver materials can be obtained from National Taiwan University Medical School because of PI's particular academic connection there.

The quantity of cell or tissue materials needed for the study will depend largely on the content of presumed retroviral specific poly(A)RNA in the cell. If there are five copies of a specific tRNA binding poly(A) RNA per cell (on the basis of estimated minimal numbers in retrovirus-transformed non-producer cells), then about 100 grams of wet cell material will be needed for a dependable analysis by our method. (See section of tRNA identification below.)

(c) RNA preparations: Total cellular RNA will be isolated mainly by the combined use of sodium dodecyl sulfate and phenol/chloroform extraction (82), either with or without prior subcellular fractionation to obtain post-nuclear supernatant for the isolation. Leukemia cells are particularly rich in the content of ribonucleases (both acid and alkaline types), which degrade RNA rapidly; RNA extraction of these cells may require direct extraction without subcellular fractionation in combination with various measures for inhibiting ribonucleases, such as heparin, natural ribonuclease inhibitors, proteinase K and/or di-ethylpyrocarbonate treatment. If necessary, guanidine thiocyanate method of RNA extraction will also be employed. Total cellular RNA will be separated into poly(A)⁺ and poly(A)⁻ RNA fractions by repeated oligo(dT)-cellulose chromatography (83). From poly(A)⁻ RNA fraction, large molecular weight RNA (including ribosomal RNAs) will be separated by precipitation in high salt solution. Transfer RNA will be isolated from high salt soluble portion of the poly(A)⁻ RNA fraction by DEAE-cellulose chromatography (79).

(d) In vitro hybridization: Total tRNA mixture and poly(A)⁺ RNA, isolated from the same cell material, will be mixed and incubated under appropriate reaction condition for allowing molecular hybridization. Our preliminary studies have revealed two technical complications in performing poly(A)⁺ RNA·tRNA hybridization. First, considerable self-aggregation of poly(A) RNA generally occurs during hybridization, thus making it difficult to estimate the size of tRNA-binding poly(A) RNA. Therefore, we will first

separate the poly(A) RNA into different size fractions (by sucrose gradient sedimentation under denaturation conditions) and perform the in vitro hybridization reaction with tRNAs. Second, poly(A)⁺ RNAs appear to be more fragile than poly(A)⁻ RNA and may be degraded considerably during hybridization. For this reason, we have recently developed a phenol emulsion procedure of hybridization, which causes minimal damage to poly(A)⁺ RNA and obtain satisfactory hybridization of poly(A)⁺ RNA and tRNAs. After hybridization, poly(A)⁺ RNA and poly(A)⁺ RNA·tRNA duplexes can be separated from unreacted free tRNA either by oligo(dT)-cellulose chromatography (with a modified chromatographic procedure) or by sucrose gradient sedimentation.

(e) Identification of tRNA: A method for qualitative and quantitative determination of tRNAs in RNA duplexes have been published (68,84a,84). In this method, RNA duplexes are isolated and heat-dissociated to release the tRNAs, which are determined by enzymatic aminoacylation reaction and subsequent analysis of the tRNA-accepted amino acids in a single column amino acid analyzer. To obtain dependable results by this method, several factors are important. First, one should use an enzyme preparation which contains all 20 aminoacyl-tRNA synthetases in sufficient activities. When carefully performed, this can obtain satisfactory enzyme preparation according to the published procedures of Yang and Novelli (79). Thus, before use an aminoacyl-tRNA synthetase preparation should be well characterized of the enzyme activities. Second, reaction condition for aminoacylation and procedures of subsequent isolation of the aminoacyl-tRNAs should be performed with precaution to minimize non-enzymatic hydrolysis of the ester bonds between amino acids and tRNAs. Different aminoacyl-tRNAs (e.g. glycyl-tRNAs vs. valyl-tRNAs) have very different rates of chemical hydrolysis at slightly alkaline pH. Third, the labeled amino acids from commercial sources should be of sufficiently high specific activity and, more importantly, of dependable quality control. In this aspect, methionine, tryptophan, phenylalanine, glutamine and asparagine will need particular attention. Also, all labeled amino acids from commercial supply are of different specific activity and not sufficiently concentrated in radioactivity. Thus, special preparations of labeled amino acids are often required. Fourth, control experiments should be carried out to differentiate non-hybridized tRNA contaminants from hybridized tRNAs. This is particularly important if the quantity of hybridized tRNAs are very small. To differentiate

the hybridized tRNA from the non-hybridized tRNA contaminants, aminoacylation is carried out with the duplex before and after heat-dissociation. Generally, a tRNA in the hybridized state does not accept amino acid in the synthetase-catalyzed reaction. Fifth, amino acid analyzer operation is critical. Some amino acids such as tryptophan may not be recovered 100% intact from the column. Under strict control of the column operation, constant recovery of each amino acid can be expected.

Based on the amino acid specific radioactivity of 20-50 Ci/mmol, the aminoacylation method is able to detect as low as $2-5 \times 10^{-15}$ mol of tRNA. This is approximately equivalent to 4×10^8 cells which contain five specific tRNA-binding poly(A) RNA molecules.

An alternative method of tRNA identification is by employing two dimensional gel electrophoresis (67) or reversed-phase chromatography (73) of the hybridized tRNA. This method has the disadvantage that the aminoacylation property of the tRNA can only be determined indirectly. However, the advantage of the method is that the increased sensitivity of the detection can be improved. Recently [γ - 32 P]-labeled ATP is available in the range of 1000-5000 Ci/mmol specificity. By using this reagent to label the 5' end phosphate of tRNA before hybridization, it would be possible to detect down to $10^{-17} \sim 10^{-18}$ mol of tRNA-binding poly(A) RNAs.

Both the aminoacylation method and the [$5'$ - 32 P]-labeled tRNA method will be applied in the present proposed research.

(f) Reverse transcription assay of primer activity: It is possible that some tRNA molecules may bind to poly(A) RNA but do not serve as primer for reverse transcription. This is true with virion 70S RNA, which contains considerable amounts of tRNA other than the primer tRNA. One simple method for differentiating primer from non-primer tRNA is to melt the RNA duplexes at different temperatures, since at least in two cases (avian and murine retroviruses) the primer tRNA is the last tRNA dissociated from viral genomic RNA by differential heat treatment. A stringent condition for hybridization reaction would also eliminate the binding of non-primer tRNA to poly(A) RNA. However, the most convincing evidence would be priming activity in the reverse transcription assay. In this assay, poly(A)RNA·tRNA duplexes will be incubated in a reaction mixture containing all four deoxynucleotide triphosphates and reverse transcriptase using incorporation of

labeled deoxynucleotide into acid-precipitable form as an indication of DNA synthesis. In the case of poly(A) RNA from human cancer cells, there will be no isologous reverse transcriptase for the assay. Purified reverse transcriptase from avian myeloblastosis virus will therefore be used in our study. We have found that this enzyme can catalyze DNA synthesis with tRNA^{Pro} primer and murine retrovirus 35S RNA as efficiently as, (if not better than) the isologous reverse transcriptase preparation.

(g) Size of primer tRNA binding poly(A) RNAs: In our preliminary experiments, we have found that poly(A) RNAs tend to aggregate considerably during hybridization. This phenomenon may complicate the study in two ways. First, hair-pin formation as well as inter-molecular duplex formation may create sites for reverse transcriptase to act, thus increasing the "background" incorporation of deoxynucleotide into DNA form (this could be corrected by appropriate control studies and modifying hybridization conditions). Second, the sizes of primer tRNA binding poly(A) RNA cannot be accurately determined after hybridization. For this reason poly(A) RNA isolated from a given cancer cell material will be first separated into different sizes by using dimethylsulfoxide-sucrose gradient sedimentation or by using preparative polyacrylamide gel electrophoresis. Gradient sedimentation has the advantage of easy recovery of RNA samples whereas gel electrophoresis gives better resolution of RNA sizes. The information about the size of primer tRNA poly(A) RNAs is important not only that it would provide a parameter for comparing different cell samples (e.g., normal vs. cancer) but also that it would indicate the possible nature of the poly(A) RNA. Above 30S size would suggest the full-length genome of endogenous retrovirus, whereas less than 30S size would suggest the subgenomic components.

(ii) Characterization of poly(A)RNA·primer tRNA binding sequences:

(a) Three kinds of experiments will be included in this project: structure determination of the complementary nucleotide sequence involved in the binding, sequence analysis of the copy DNA made from tRNA-primed reverse transcription, and (especially if the copy DNA is of "short-stop" nature), the primer activity of the copy DNA for the parent poly(A) RNA to detect the possible redundant sequence. These studies are the natural

extension of the experiments listed in previous (i) section, especially if certain human cancer cells would be found to contain specific poly(A) RNA molecules with "primer tRNA" binding activity.

(b) RNA sequence analysis: Priming function of the hybridized tRNA is provided by a 3' OH nucleotide which is attached to the template by hydrogen bonds. The tRNA^{trp} binding to avian retrovirus genomic RNA and the tRNA^{pro} binding to murine retrovirus genomic RNA all involve a 15-20 base sequence from 3' end of the tRNA molecule. Thus, to determine the primer property, one has to find out if a given "primer tRNA" binds to poly(A) RNA of human cells in a similar fashion. [³²P]-labeled tRNA will be prepared, separated by reversed-phase chromatography, and used to form duplex with the poly(A) RNA. The duplex will be isolated, subjected to ribonuclease A and T1 digestion under conditions which do not hydrolyze the duplex RNA portion. The undigested [³²P]-labeled tRNA fragment can then be analyzed by oligonucleotide finger-printing technique. By comparison with the total sequence information of whole tRNA molecules, it would be possible to locate the stretch of the tRNA sequence involved in the binding. Standard methods for RNA sequence analyses have been well established (85,86).

The site of primer tRNA binding is known to be near the 5' end of retrovirus genomic RNAs. Thus, it would be important to determine if such is the case with "primer tRNA" binding poly(A) RNAs of human cancer cells. For this reason, the experimental approach of Taylor and Illmensee (71) will be applied. Poly(A) RNA will be partially fragmented by mild alkaline treatment. Different size fragments with 3' poly(A) ends will be isolated by oligo(dT)-cellulose chromatography. Primer tRNA binding will be performed with these different size poly(A) RNA fragments.

(c) DNA sequence analysis: Recently, the DNA sequencing has become possible with the application of Maxam and Gilbert's method (87). This has been elegantly applied by Haseltine and co-workers in the study of 5' end sequence of RNA tumor viruses. Thus, it will be possible to characterize the in vitro synthesized copy DNA from reverse transcription of poly(A) RNA-primer tRNA. In this study, cDNA products will be first separated by gel electrophoresis and the defined band will then be used for the analysis. DNA sequencing will be performed with the initial help from Dr. Dorothy Skinner

(Biology Division, ORNL), who has obtained excellent results with her crab satellite DNA studies by employing Maxam-Gilbert's method.

(d) Molecular hybridization: Recent experimental evidences have indicated that subgenomic size mRNAs of RNA tumor viruses may contain a "leader" sequence which is derived from 5' end of the viral genome. The short cDNA from tRNA-primed synthesis is complementary to the 5' end "leader" sequence and, therefore, able to hybridize to viral subgenomic RNAs. This will be examined with the cDNAs obtained from tRNA-primed poly(A) RNAs of human cells, namely, whether or not a given cDNA preparation can hybridize with different size poly(A) RNAs of the same cell. Furthermore, by using presumably "leader" sequence-specific cDNAs from different cells and cancers, it would be possible to determine if "leader" sequences are the same or different in normal vs. cancer cells or in different cancer cells. One convenient method for such a study is molecular hybridization. For this, a phenol emulsion technique modified from the PERT method of Kohne, Levinson and Byer (88) has been used in this laboratory with satisfactory results.

(e) Priming properties of cDNA complementary to the "leader" sequences: A redundant sequence of about 20 nucleotides length is present both at the 5' terminus and at the 3' poly(A)-adjoining region of RNA tumor virus genomic RNA (89,90). This provides a molecular basis for continuous reverse transcription from the 5' end to the 3' end of viral genomic RNA. Thus, copy DNA complementary to the "leader" sequences should be able to hybridize with the 3' end of viral genomic RNA at the redundant sequence. In the present study of intracellular "primer tRNA"-binding poly(A) RNAs of human cancer cells, it would be interesting to examine whether such redundant sequences are present or not. This can be most conveniently demonstrated by testing the priming property of the "leader" sequence specific cDNA for the parent poly(A) RNA fraction. Such experiments will not only answer the question of redundant sequences but also provide a means to synthesize the full-length copy DNA of the "primer-tRNA" binding poly(A) RNAs.

(iii) Purification of "primer tRNA"-binding poly(A) RNAs from human cancer cells:

(a) General experimental design: In principle, the specific poly(A) RNA can be separated from other poly(A) RNA species by the use of particular tRNA molecules attached to solid matrix in affinity chromatography. In practice, this will involve purification of the particular tRNA in large quantity, conjugation of the tRNA to the Sepharose gel, and affinity chromatographic procedures. Variation of the purification procedure will include conventional RNA separation methods using binding of the labeled tRNA for assaying the separated RNA fraction, and the "R loop" formation between long copy DNA and the poly(A) RNAs for subsequent separation by cesium chloride density gradient centrifugation.

(b) Large-scale purification of tRNA: The primary structure of an amino acid tRNA is generally well-conserved, or even identical from animal species to animal species. For example, tRNA^{trp} from mouse serves as primer tRNA for avian RNA tumor viruses as well as tRNA^{trp} from chicken cells. Since human cell tRNAs cannot be obtained in large quantities, the purification experiment will use tRNA mixture prepared from beef liver. Although available from commercial sources, 20 grams of beef liver tRNA will cost a considerable amount of money. We have recently devised a simple and economical method for preparing tRNA from beef liver (Yang, *et al.*, unpublished observation). With the method, 20 grams of tRNA may be prepared as a starting material for purification of individual tRNA species. tRNA purification will be carried out by successive chromatography in Sepharose gel (hydrophobic adsorption chromatography) and in reversed-phase RPC-5 and RPC-6 columns. Successive chromatography in the same RPC column with different buffer pH and salt gradient would also be helpful in the separation. Purity of the isolated tRNA species will be assessed by the amino acid accepting activity.

(c) tRNA-Sepharose gel preparation: tRNA-Sepharose gel has been employed mainly for the purification of aminoacyl-tRNA synthetases (e.g., 91-93). In these studies, tRNA was attached to Sepharose 4B through adipic acid dihydrazide or hydrazine linkage. For the present work, similar methods may be used for preparation of affinity gel with purified

tRNA samples. The use of linkage between Sepharose gel matrix and tRNA is important particularly for poly(A) RNA separation purposes, because the conjugation of tRNA is at the 3' OH end. This may allow the 3' end region of the conjugated tRNA to freely interact with the poly(A) RNAs during affinity chromatography.

tRNA-Sepharose gel will be used for affinity chromatography of poly(A) RNAs isolated from cancer tissues, which may be obtained in large quantities through collection of surgically removed specimens. Conditions of chromatography will follow those optimal for poly(A) RNA-tRNA interaction.

(d) Alternative methods of specific poly(A) RNA isolation will include the use of R-loop formation with subsequent cesium chloride density gradient centrifugation, the use of conventional nucleic acid separation methods, and the appropriate application of oligo(dT)-cDNA-cellulose preparations from human cancer cells and from human normal cells (94). Assay of separated poly(A) RNA fractions by these methods will utilize the [5',³²P]-labeled "primer tRNA" for the binding.

(iv) Biological Characterization of "Primer tRNA"-Binding Poly(A) RNAs (Brief Prospectus)

(a) Messenger RNA activity of the isolated "primer tRNA"-binding poly(A) RNAs can be determined either by micro-injection into Xenopus laevis oocytes (95) or by cell-free protein synthesis in the micrococcal nuclease-treated cell lysate systems (e.g. 96). Ability of the RNA to stimulate amino acid incorporation into protein can be quantitatively determined. Protein products can then be analyzed by two dimensional isoelectric focusing/polyacrylamide electrophoresis gel method (97,98). Immune-precipitation tests may also be carried out with the labeled cell-free synthesized protein and the sera of cancer patients at various stages of disease; this may provide a means for detecting specific immune response against human cancers.

(b) Application of copy DNAs representing "leader" sequences, 3' end sequence and total poly(A) sequence will be possible in pathological and clinical studies, especially in relation to morphological types of a given cancer and disease progression or regression. Such studies will

involve molecular hybridization to detect the expression of these sequences into RNA molecules.

(c) Gene location may be studied by using the methods of restriction endonuclease, agarose gel electrophoresis with Southern's blotting technique (99) and labeled purified poly(A) RNA as the probe (100).

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KEY PERSONNEL

A. Supported Personnel

Wen K. Yang, M.D., Ph.D., Principal Investigator (25%)

Will direct all aspects of the proposed research and be responsible for implementation of an awarded contract through the NCI-DOE interagency agreement. With academic background in medicine and biochemistry, Dr. Yang's research experience includes riboflavin metabolism in human and experimental hepatocarcinomas, host defense mechanisms in Chagas' disease, nucleic acid enzymology and biochemistry of tRNAs and protein biosynthesis. The methods that he devised for tRNA isolation and synthetase preparation from mammalian tissues and cells have been widely applied. For the past seven years his major research has been on host cell control of retrovirus expression, particularly the mechanism of Fv-1 locus restriction of murine ecotropic retroviruses and the primer tRNA functions.

David L. Hwang, Ph.D. Biochemist (100%) Nucleic acid enzymology and tRNA identification. With past training in protein purification and characterization, Dr. Hwang has been working with rRNA·tRNA interaction for the past two years, and has accumulated considerable experience related to this aspect of the study.

Daniel J. Price, B.S. Biochemistry Assistant (100%). Mr. Price will be responsible for preparation and isolation of RNA from human cancer cells and tissues and assistance of various biochemical operations.

Claude D. Stringer, M.S. Biochemistry Research Assistant (50%). Mr. Stringer operates the amino acid analyzer and will be responsible for identification of labeled amino acids charged to tRNAs. He will also check the purity of commercially labeled amino acids to be used for the aminoacylation.

Den-Mei Yang, B.A. Tissue Culture and Cell Biology Research Assistant (50%). Ms. Yang has over four years of experience in tissue culture and retrovirus research. She will be responsible for large-scale propagation of human cancer cells in vitro, maintenance of the cell lines and assay of virus infection.

B. Personnel Supported by Other Sources

Fred C. Hartman, Ph.D. Biochemist. Collaboration on utilization of amino acid analyzer for identification of charged labeled aminoacyl-tRNAs and peptide analysis.

Davis K. Lin, M.D., Ph.D. (University of Tennessee-Memorial Hospital). Collaboration on characterization of cell-free synthesized proteins and assistance in securing biopsy cancer materials.

G. David Novelli, Ph.D. Biochemist. Collaboration and consultation on large-scale purification of individual tRNAs by reversed-phase chromatography.

Grace Shiao, M.S., Ph.D. Candidate (University of Tennessee-Oak Ridge Graduate School of Biomedical Sciences). Full-time investigation on poly(A)RNA-tRNA hybridization by employing human breast cancer cells.

Chin-Yih Ou, M.S. Graduate Student (University of Tennessee-Oak Ridge Graduate School of Biomedical Sciences). Full-time research participation in the expression of cell-genome integrated retroviral genes.

In addition, this laboratory generally attracts research participation by undergraduate premedical students (average two per year) from the Oak Ridge Science Semester programs of the Great Lakes College Association, Southern College and University Union and Oak Ridge Associated Universities-Summer Research Fellowship Program.

PROPOSED BUDGET

"Retroviral Genetic Expression in Human Cancers: Analysis by
Primer tRNA Binding Approach (Yang)

Proposed Addition to NCI-DOE Interagency Agreement

	<u>FY 1979</u>		<u>FY 1980</u>		<u>FY 1981</u>	
	<u>01</u>		<u>02</u>		<u>03</u>	
	<u>MY</u>	<u>\$</u>	<u>MY</u>	<u>\$</u>	<u>MY</u>	<u>\$</u>
W. K. Yang	0.25		0.50		0.50	
D. L. Hwang	<u>1.00</u>		<u>1.00</u>		<u>1.00</u>	
Total Ph.D.	1.25		1.50		1.50	
D. J. Price	1.00		1.00		1.00	
C. D. Stringer	0.50		0.50		0.50	
D. M. Yang	<u>0.50</u>		<u>0.50</u>		<u>0.50</u>	
Total Technicians	2.00		2.00		2.00	

DIRECT COSTS:

Salaries		59,200		67,700		71,700
Travel		1,200		1,200		1,200
Materials & Supplies		17,000		26,000		30,000
Shop & Maintenance		800		1,200		1,200
Glassware Washing (Kitchen)		<u>6,000</u>		<u>7,000</u>		<u>7,700</u>
Total	(3.25)	<u>\$84,200</u>	(3.5)	<u>\$103,100</u>	(3.5)	<u>\$111,800</u>

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FACILITIES AND EQUIPMENT:

The 2923 sq. ft. of space occupied by this group includes (a) two biochemical laboratories with necessary facilities for advanced biochemical research in nucleic acid enzymology and protein biosynthesis; (b) a tissue culture facility sufficient for specific work of RNA tumor viruses with respect to biohazard protection and large-scale cell growth; (c) a 4°C cold room; (d) a walk-in 37°C incubator and (e) liquid nitrogen freezers. One-third of the space and facility is proposed for use in the present proposed research.

In addition, highly sophisticated amino acid analyzer and peptide sequencer facilities are generously available for this research from Dr. Fred Hartman's laboratory (Biology Division, ORNL).

EXPERIMENTS WITH HUMAN SUBJECTS - EXPERIMENTS WITH RECOMBINANT DNA

No such experiments are anticipated and will not be carried out in the present proposed research.

RELATED RESEARCH ACTIVITIES AND PARTICULAR CONSIDERATION:

Currently there are two budgetary supports for this laboratory: A Department of Energy-supported "Carcinogen-Cell Genome Interaction" project and an NCI-supported "Host Cell Control of Retrovirus Expression" project.

The DOE-supported project involves research activities related to the effect of environmental carcinogens on integration of exogenous oncogenic (retroviral) information into cell genome and also on enzyme mechanisms of cell DNA replication and repair.

The NCI-supported project is through an interagency agreement. The major research activity centers on "The mechanism of Fv-1 locus restriction of murine leukemia viruses", a joint research effort involving R. W. Tennant's group (Cell Biology and Virology) and this laboratory (Biochemistry). Included in this project is also a minor activity on the identification of primer tRNAs of RNA tumor viruses. Because of the budgetary limitations, the distribution of the Y01 CP6-0500 budget to the primer tRNA research is currently 1.00 man year (FY 1978). With an anticipated decline of the Y01 CP6-0500 budget, there will be a further curtailment on the primer tRNA research portion in FY 1979 to 0.5 man year. The primer tRNA research for the Y01 CP6-0500 contract mainly concerns identification of primer tRNAs in known retrovirus isolates and possible cellular origin of tRNA binding sequences (see Background Section). The results from these works have contributed to the idea for generating the present research proposal; the two projects do not duplicate in research content, nor in manpower effort. However, if the present research proposal is awarded with a contract through NCI-DOE interagency agreement, the primer tRNA research activity in the Y01 CP6-0500 should nevertheless be terminated in FY 1980. This is reflected in the budget proposal in which the principal investigator's effort will increase from 0.25 MY in FY 1979 to 0.50 MY in FY 1980.

APPENDIX I

Curriculum Vitae

WEN-KUANG YANG

Born [REDACTED] China, came to U.S. in 1963, obtained U.S. permanent residency in 1969, and became a U.S. citizen in 1974; married to [REDACTED].

EDUCATIONAL AND PROFESSIONAL BACKGROUND:

[REDACTED]

1961-1962 Rotating Internship, National Taiwan University Hospital, Taipei, Taiwan

[REDACTED]

1963 Second Lieutenant Medical Officer, Chinese Air Force

[REDACTED]

1966-1968 Damon Runyon Fellow for Cancer Research, Visiting Investigator, Biology Division, Oak Ridge National Laboratory

1968-1973 Staff Biochemist, Carcinogenesis Program, Biology Division, Oak Ridge National Laboratory

1973-pres. Group Leader, Enzymology of Carcinogenesis Group, Biology Division, Oak Ridge National Laboratory

HONORS, MEMBERSHIPS AND OTHER POSITIONS:

Frank Hsu Scholarship for Natural Sciences (1956)

Chong-Mei Medical Scholarship (1958)

Sigma Xi Award, Tulane (1967)

Damon Runyon Memorial Fellowship for Cancer Research (1966-1968)

Full member, Sigma Xi Society (1966-present)

Active member, Gerontological Society (1970-present)

Active member, American Association for Cancer Research (1972-present)

Active member, American Society of Biological Chemists (1973-present)

Member, American Association for Advancement of Science (1965-present)

Lecturer in Biomedical Sciences, University of Tennessee-Oak Ridge Graduate School of Biomedical Sciences (1969-present); teach advanced courses "Biochemical Concepts in Medical Sciences" (3 credit hours) and "Medical Genetics" (3 credit hours) once every two years.

Regular reviewer of scientific papers for Cancer Research, Archives of Biochemistry and Biophysics, Journal of Biological Chemistry and Journal of National Cancer Institute

Yang, W. K. Riboflavin metabolism in liver diseases, especially hepatoma, 59 pp. B. M. Thesis, National Taiwan University College of Medicine (1961).

Yang, W. K. and Sung, J. L. Riboflavin metabolism in liver diseases, especially hepatoma I. Blood flavin levels in liver diseases, II. Flavin adenine dinucleotide (FAD) loading on the liver disease patients, III. Flavin contents of the liver and other tissues of p-dimethylaminoazobenzene (DMAB)-fed rat and human livers, IV. Enzymatic splitting and synthesis of FAD in the DMAB induced rat liver carcinoma tissues, V. Clinical studies of the serum enzymatic splitting of FAD. *Journal of Formosan Medical Assoc.* 65: 282-286, 287-293, 294-298, 299-305, 306-311 (1966).

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- I. C. Hsu and W. K. Yang. DNA transfection of ecotropic murine leukemia viruses in mouse cell cultures. *Cancer Res.* 37: 1709-1714 (1977).
- D. W. Fountain and W. K. Yang. Isolectins from soybean (Glycine max.) *Acta Bioch. Bioph.* 492: 176-185 (1977).
- D. W. Fountain, D. E. Foard, W. D. Replogle and W. K. Yang. Lectin release by soybean seeds. *Science* 197: 1185-1187 (1977).
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- D. L. R. Hwang, W. K. Yang, D. E. Foard and K.-T. D. Lin. Rapid release of protease inhibitors from soybeans. Immunochemical quantitation and parallels with lectins. *Plant Physiol.* 61: 30-34 (1978).
- W. K. Yang, D. L. R. Hwang, J. O. Kiggans, D. M. Yang, C. D. Stringer, D. J. Moore, and F. C. Hartman. In vitro association of selective tRNA species with 28S RNA of mouse cells. *Biochem. Biophys. Res. Comm.* 80: 443-450 (1978).
- R. W. Tennant, W. K. Yang, R. J. Rascati, I. C. Hsu and A. Brown. Specific cell RNA mediators and the mechanism of Fv-1 gene restriction. *Oji International Seminar on Genetic Aspects of Friend Virus and Friend Cells.* Academic Press, N. Y., in press (1978).

- I. C. Hsu, W. K. Yang, R.W. Tennant and A. Brown. Transfection of Fv-1 permissive and restrictive mouse cells with integrated DNA of murine leukemia viruses. Proc. Nat. Acad. Sci. U.S.A. 75:1451-1455 (1978).
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- D. L. R. Hwang, W. K. Yang, J. O. Kiggans, Jr. and C. D. Stringer. In vitro binding of selective tRNAs to 18S and 28S ribosomal RNAs of mouse cells. Nucleic Acid Res. submitted (1978).
- S. M. Huang and W. K. Yang. On the DNA polymerase activity of rat spleen. I. Partial purification and characterization of three DNA polymerases. Arch. Biochem. Biophys. submitted (1978).
- S. M. Huang and W. K. Yang. On the DNA polymerase activity of rat spleen. II. Age-associated decrease of DNA polymerase activity. Arch. Biochem. Biophys. submitted (1978).
- W. K. Yang, L. R. Boone, D. M. Yang, I. C. Hsu, R. W. Tennant and A. Brown. Infectious activity of unintegrated proviral DNAs of murine N-tropic and B-tropic retroviruses. Effects of Fv-1 locus restriction. Proc. Nat. Acad. Sci. U.S.A. Submitted (1978).

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Curriculum Vitae

Name: David L. Hwang

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Home Phone: [REDACTED]

Business Address: Biology Division, Oak Ridge National Laboratory
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Business Phone: (615) 483-8611, Ext. 3-7128

Current Position: Postdoctoral Investigator, Cancer & Toxicology Program,
Biology Division, Oak Ridge National Laboratory,
Oak Ridge, TN 37830

Date of Birth: [REDACTED]

Place of Birth: [REDACTED] Republic of China (U.S. Permanent Resident)

Martial Status: [REDACTED]

Education:

Professional Experience:

Laboratory Instructor and Research Assistant, National Chung-Hsing
University, Taiwan, 1968-70

Instructor, Taichung Agricultural High School, Taiwan, 1970-71

Teaching Assistant, South Dakota State University, 1971-73

Teaching and Research Assistant, University of Nebraska, 1973-75

Postdoctoral Investigator, Molecular & Cellular Sciences Program,
Biology Division, Oak Ridge National Laboratory, 1975-76

Postdoctoral Investigator, Cancer & Toxicology Program, Biology
Division, Oak Ridge National Laboratory, 1977-pres.

Thesis:

M.S. - "Effects of Mimosine on Various Reactions of Amino Acids"
South Dakota State University, 1973

Ph.D. - "Isolation and Characterization of Growth Inhibitors from
Great Northern Beans" University of Nebraska, 1975

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David L. Hwang

List of Publications:

D. L. Hwang, D. E. Foard and C. H. Wei. A Soybean Trypsin Inhibitor-Crystallization and X-ray Crystallographic Study. *J. Biol. Chem.* 252: 1099, 1977.

D. L. Hwang, K. T. D. Lin, W. K. Yang and D. E. Foard. Purification, Partial Characterization and Immunological Relationships of Multiple Low Molecular Weight Protease Inhibitor of Soybean. *Biochim. et Biophys. Acta* 495: 369, 1977.

D. L. Hwang, W. K. Yang, K. T. D. Lin, and D. E. Foard. Rapid Release of Protease Inhibitors from Soybeans-Immunochemical Quantitation and Parallels with Lectins. *Plant Physiol.* 61: 30, 1978.

W. K. Yang, D. L. Hwang, J. O. Kiggans, Jr., D. M. Yang, C. D. Stringer, D. J. Moore and F. C. Hartman. In Vitro Association of Selective tRNA Species with 28S RNA of Mouse Cells. *Biochem. Biophys. Res. Commun.* 80: 443, 1978.

D. L. Hwang, W. K. Yang, J. O. Kiggans, Jr., and C. D. Stringer. In Vitro Binding of Selective Transfer RNAs to 18S and 28S Ribosomal RNAs of Mouse Cells. *Nucleic Acid Res.*, in press, 1978.

D. E. Foard, D. L. Hwang, W. K. Pao and W. K. Yang. Quantitative Alteration of High Methionine-Half-Cystine Proteins in Soybeans: Analysis of Genetic and Environmental Influences by a Radioimmunoassay. (submitted).

D. L. Hwang, D. J. Price, D. M. Yang, and W. K. Yang. In Vitro Hybridization of Selective Transfer RNA Species with 18S and 28S Ribosomal RNAs of Various Animal Cells. (submitted).

D. L. Hwang, K. T. D. Lin, W. K. Pao and D. E. Foard. Purification of Soybean Protease Inhibitors by Affinity Chromatography. (Manuscript in preparation).

D. E. Foard, D. L. Hwang, W. K. Pao, and K. T. D. Lin. Analysis of Protease from Human Urine, Ascites and Pleural Effusion by Affinity Chromatography. (Manuscript in preparation).

Abstracts:

D. L. Hwang and D. E. Foard. Protein Fractions with High Content of Sulfur-Containing Amino Acids in Soybean Seeds. American Society of Plant Physiologists (AIBS) New Orleans, LA, May 30-June 4, 1976.

D. L. Hwang and W. K. Yang and D. E. Foard. Immunological Studies of Low Molecular Weight Soybean Proteinase Inhibitors. 11th Federation of European Biochemical Societies. Copenhagen, Denmark, August 14-19, 1977.

J. O. Kiggans, Jr., D. L. Hwang and W. K. Yang. In Vitro Binding of Selective tRNA Species to Cellular and Murine Leukemia Virus-Coded RNA. American Association of Cancer Research, Washington, D. C. April 5-18, 1978.

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David L. Hwang

D. L. Hwang, J. O. Kiggans, Jr., D. J. Price, D. M. Yang and W. K. Yang. In Vitro Hybridization of Selective Transfer RNA Species with 18S and 28S Ribosomal RNAs of Various Animal Cells. American Society of Biological Chemist/American Association of Immunologist. Atlanta, GA, June 4-8, 1978.

W. K. Yang, D. L. Hwang, F. C. Hartman, D. J. Price, J. O. Kiggans, Jr., C. D. Stringer and D. M. Yang. Primer tRNA Binding of Cellular RNAs from Retrovirus-Infected-Transformed and Noninfected Cells. Proc. RNA Tumor Virus Meeting, Cold Spring Harbor Laboratory, May 24-28, p. 82, 1978.

D. E. Foard, D. L. Hwang, W. K. Pao, and W. K. Yang. Quantitative Alteration of High Methionine-Half-Cystine Proteins in Soybeans: Analysis of Genetic and Environmental Influence by a Radioimmunoassay. International Symposium on Seed Protein Improvement in Cereals and Grain Legumes. Neuherberg, Federal Republic of Germany, September 4-8, 1978.

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Born:

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Education

[REDACTED]

Teaching Experience

General Chemistry Laboratory - 1960-62 - Tennessee Technological University.
Organic Chemistry Laboratory - 1961 - Tennessee Technological University.
Qual. & Quan. Chemistry Laboratory - 1961 - Tennessee Technological University.

Research Experience (1971 to present)

Research Associate, Biology Division, Oak Ridge National Laboratory
Extensive experience in organic synthesis and protein chemistry, total responsibility for the operation and maintenance of an amino acid analyzer and protein sequencer.

Scientific Societies:

American Chemical Society

Publications

- Norton, I. L., Pfuderer, P., Stringer, C. D. and Hartman, F. C. (1970), Isolation and Characterization of Rabbit Muscle Triose Phosphate Isomerase. Biochemistry 9, 4952.
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