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Carter, Linda Jane

SELECTIVE ALTERATION OF SNYDER-THEILEN FELINE SARCOMA VIRUS TRANSFORMING GENE (V-FES) INTEGRATION IN CHEMICALLY-TREATED HUMAN FIBROBLASTS

The Ohio State University

Ph.D. 1984

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SELECTIVE ALTERATION OF SNYDER-THEILEN FELINE SARCOMA VIRUS TRANSFORMING GENE (v-fes) INTEGRATION IN CHEMICALLY-TREATED HUMAN FIBROBLASTS

DISSertation

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

Linda Jane Carter, B.S., M.S.

* * * * *

The Ohio State University

1984

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Linda Jane Carter

1984
Dedicated to the memory of my father, Emil Frick, for his simple honesty and love of nature.
Acknowledgements

I am grateful for the help and suggestions of many people given to me throughout this research project. I am indebted to Dr. Genoveffa Franchini for providing me with subclones of the v-fes gene and to Dr. H.G. Kung for valuable assistance with the dot-blot hybridization methodology. Dr. John Reeve and Mr. Paul Hamilton generously contributed a culture of E. coli containing pBR322 plasmid and volunteered their time to demonstrate plasmid purification and radiolabelling. Dr. Deborah Parris offered valuable recommendations and advice regarding hybridization procedures. Mrs. Ann Elliot was a constant source of encouragement and often assisted with co-carcinogenesis transformation experiments. She also provided the Snyder-Theilen feline sarcoma virus used for transformation. I am especially appreciative of the opportunity given to me by Dr. James R. Blakeslee, Jr. to perform this research. I value his understanding of my independence as a researcher and thank him for his time and worthwhile suggestions. I would also like to thank Ms. Virginia Stump for her gracious time and patience in typing this dissertation.
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Studies in Chemical-Viral Interactions. Dr. James R. Blakeslee, Jr.
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<td>A-AAF</td>
<td>N-acetoxy-N-2-Acetylaminofluorene</td>
</tr>
<tr>
<td>AAF</td>
<td>Acetylaminofluorene</td>
</tr>
<tr>
<td>ABl</td>
<td>Alflatoxin B₁</td>
</tr>
<tr>
<td>Ara-C</td>
<td>1-β-D-arabino-furanosylcytosine</td>
</tr>
<tr>
<td>ASV</td>
<td>Avian sarcoma virus</td>
</tr>
<tr>
<td>AZA</td>
<td>5-azacytidine</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BUdR</td>
<td>5-bromodeoxyuridine</td>
</tr>
<tr>
<td>BP</td>
<td>Benzo[a]pyrene</td>
</tr>
<tr>
<td>C-DNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CM</td>
<td>Complete Medium</td>
</tr>
<tr>
<td>D-550</td>
<td>Detroit 550 - CCL109 human foreskin fibroblast cells</td>
</tr>
<tr>
<td>DDT</td>
<td>1,1,1-trichloro-2,2-bis(β-chlorophenyl)ethane</td>
</tr>
<tr>
<td>DEN</td>
<td>Diethylnitrosamine</td>
</tr>
<tr>
<td>DHEA</td>
<td>Dehydroepiandrosterone</td>
</tr>
<tr>
<td>7,12-DMBA</td>
<td>7,12-Dimethylbenzantracene</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EA</td>
<td>Early Antigen</td>
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EBV    Epstein-Barr Virus
EC     Embryonal Carcinoma
EGF    Epidermal growth factor
EMS    Ethyl methane sulfonate
Epi-Br 16α-Bromo-epiandrosterone
EtBr   Ethidium bromide
FBS    Fetal bovine serum
FFU    Focus-forming unit
GA-FeLV Feline leukemia virus - Gardner-Amstein strain
G6PDH  Glucose-6-phosphate dehydrogenase
gs     group-specific
HSV    Herpes Simplex Virus
HU     Hydroxyurea
IUdR   5-Iododeoxyuridine
KT-FeLV Feline leukemia virus - Kawakami-Theilen strain
LB     Luria-Bertani Medium
L-dopa L-β-3,4-dihydroxyphenylalanine
MA     Membrane Antigen
MCA    Methylcholanthrene
7-MG   7-Methylguanine
**LIST OF ABBREVIATIONS** (Continued)

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<td>Mitomycin C</td>
</tr>
<tr>
<td>MMH</td>
<td>Mono-methyl hydrazine</td>
</tr>
<tr>
<td>MMS</td>
<td>Methyl methane sulfonate</td>
</tr>
<tr>
<td>MMTV</td>
<td>Mouse mammary tumor virus</td>
</tr>
<tr>
<td>MNNG</td>
<td>N-methyl-N' -nitro-N-nitrosoguanidine</td>
</tr>
<tr>
<td>MNU</td>
<td>Methyl nitrosourea</td>
</tr>
<tr>
<td>Mol. Wgt.</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>MuLV</td>
<td>Murine leukemia virus</td>
</tr>
<tr>
<td>MSV</td>
<td>Murine sarcoma virus</td>
</tr>
<tr>
<td>NMU</td>
<td>Nitroso-methylurea</td>
</tr>
<tr>
<td>4-NQO</td>
<td>4-Nitroquinoline-1-oxide</td>
</tr>
<tr>
<td>O\textsuperscript{6}MG</td>
<td>O\textsuperscript{6}-Methylguanine</td>
</tr>
<tr>
<td>PANA</td>
<td>Phenyl-\textit{\textalpha{}} -Naphthylamine</td>
</tr>
<tr>
<td>PS</td>
<td>Propane sultone</td>
</tr>
<tr>
<td>RPA</td>
<td>12-0-Retinoylphorbol-13-acetate</td>
</tr>
<tr>
<td>RSV</td>
<td>Rous sarcoma virus</td>
</tr>
<tr>
<td>SA</td>
<td>Simian adenovirus</td>
</tr>
<tr>
<td>SCE</td>
<td>Sister chromatid exchange</td>
</tr>
<tr>
<td>SDMH</td>
<td>Symmetrical dimethyl hydrazine</td>
</tr>
<tr>
<td>SGF</td>
<td>Sarcoma growth factor</td>
</tr>
<tr>
<td>SSC Buffer</td>
<td>2X</td>
</tr>
<tr>
<td>--------------------</td>
<td>----</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.3M</td>
</tr>
<tr>
<td>Nacitrate</td>
<td>0.03M</td>
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ST-FeSV            Feline sarcoma virus – Snyder-Theilen strain

SV40               Simian virus 40

TE Buffer          0.01 N Tris-HCl
                   0.001M EDTA

TNE Buffer         0.01M Tris-HCl
                   0.1M NaCl
                   0.001M EDTA

TPA                12-0-tetradecanoyl-phorbol-13-acetate

UDMH               Unsymmetrical dimethyl hydrazine

VCA                Viral capsid antigen
LIST OF SOURCES FOR MATERIALS

Aldrich Chemical Company
   Milwaukee, WI

American Type Culture Collection
   Rockville, MD

Amersham
   Arlington Heights, IL

Beckman Instruments, Inc.
   Palo Alto, CA

Bethesda Research Laboratories, Inc.
   Gaithersburg, MD

Biorad
   Richmond, CA

Boehringer Mannheim
   Indianapolis, IN

Branson Sonic Power Co.
   Danbury, CT

Calbiochem-Behring Corp.
   San Diego, CA

DIFCO Laboratories
   Detroit, MI

Eastman Kodak Company
   Rochester, NY

Fotodyne Incorporated
   New Berlin, WI

FMC Corporation, Marine Colloids Division
   Rockland, ME
LIST OF SOURCES FOR MATERIALS (Continued)

GIBCO Laboratories
   Grand Island, NY

ISCO
   Lincoln, NE

LKB Instruments, Inc.
   Rockville, MD

Kapak Corp.
   Bloomington, MN

Miles Laboratories, Inc.
   Elkhart, IN

Millipore Corporation
   Bedford, MA

New England Nuclear - Dupont
   Boston, MA

Packard Instrument Company, Inc.
   Downers Grove, IL

Pharmacia Fine Chemicals
   Piscataway, NJ

P.L. Biochemicals
   Milwaukee, WI

Precision Scientific Co.
   Chicago, IL

Rainen Instrument Co., Inc.
   Woburn, MA

Sigma
   St. Louis, MO

Sterile Systems, Inc.
   Logan, UT
LIST OF SOURCES FOR MATERIALS (Continued)

Whatman Limited, England
Supplier - American Scientific Products
McGaw Park, IL
INTRODUCTION

This research study investigated the interaction of a virus (specifically feline sarcoma virus) and chemicals in the oncogenic process, sometimes referred to as syn-carcinogenesis or co-carcinogenesis. Chemical carcinogens may enhance or inhibit oncogenic virus expression including transformation, depending upon such variables as: 1) the in vitro or in vivo system involved, 2) the type of transforming virus used for infection (RNA or DNA virus) and whether it is exogenously or endogenously transmitted, 3) the time of chemical treatment relative to virus infection, 4) the concentration of chemical used in the treatment procedure, and 5) the mode of action of the carcinogen or tumor promoter used for treatment.

The basis for this study was the discovery that chemical carcinogens inhibited the transformation of human cells by feline sarcoma virus - Snyder-Theilen strain (ST-FeSV) at critical time periods. Blakeslee and Milo (1978) evaluated the ability of various chemicals to alter focus induction by ST-FeSV in a line of human foreskin fibroblast cells (Detroit 550 - CCL109). The carcinogens, benzo[a]-pyrene (BP), N-acetoxy-N-2-acetylaminofluorene (A-AAF), and aflatoxin B₁ (AB₁), consistently inhibited focus induction at various time periods ranging from 2 to 24 hrs either previous to or after infection
with ST-FeSV. In contrast, treatment with pyrene (noncarcinogenic analog of BP) both before and after virus infection over a 48 hr time period had no effect on focus formation. The Detroit 550 (D-550) line of human fibroblast cells are nonproductive, but allow for partial gene expression of ST-FeSV as indicated by reverse transcriptase, focus induction, and feline oncornavirus-associated cell membrane antigen on the cell surface (FOCMA-S) (Blakeslee and Milo, 1978; Blakeslee et al., 1980). Chemicals appeared to be selectively altering the transforming ability (encoded by v-fes) of the virus. When a broad range of chemicals were screened using the human fibroblast-ST-FeSV system as a co-carcinogen assay, carcinogens significantly inhibited ST-FeSV focus induction when administered at 2 hrs after virus infection (Blakeslee et al., 1983a; 1983b; 1983c). Non-carcinogens had no significant effect on transformation when added at this time. Some of the classes of chemicals screened included the aromatic amines, polycyclic aromatic hydrocarbons, hydrazines and various other compounds including AB_1, amosite asbestos, acetone, formalin, Triton X-100, and various fuels, including both shale and petroleum-derived forms (Blakeslee et al., 1983a; 1983b; 1983c).

The mechanism of inhibition of ST-FeSV transformation by chemical carcinogens was not determined. Since the inhibitory effects of carcinogens were observed when chemicals were added 2 hrs after virus infection it was suggested that carcinogens might interfere with proviral or v-fes synthesis and integration (Blakeslee and Milo, 1978; Blakeslee et al., 1983c). Evidence for interference with these
viral processes comes from studies of avian sarcoma and murine leukemia viruses. In these systems proviral DNA is apparently synthesized and transported from the cytoplasm into the nucleus between 6 to 24 hrs after viral infection (Varmus et al., 1974; Gianni et al., 1975).

This research study was done to determine whether chemicals affected ST-FeSV proviral synthesis and integration in human fibroblast cells. Two different methods of approach were used. Both involved the preparation of a radiolabelled probe for detecting ST-FeSV proviral DNA in the cytoplasm and nuclear DNA of chemically-treated and non-treated human fibroblast cells (D-550). The first approach involved the following steps: 1) the culture and purification of the Kawakami-Theilen strain of feline leukemia virus (KT-FeLV); 2) purification of viral RNA from KT-FeLV; and 3) preparation of a radiolabelled complementary DNA (C-DNA) copy of KT-FeLV RNA in an exogenous in vitro reaction using avian myeloblastosis virus reverse transcriptase. Because of the limited specificity of a C-DNA probe to KT-FeLV for ST-FeSV transforming sequences, a second method of approach was used.

The availability of clones containing the v-fes gene of ST-FeSV improved the specificity of detection of FeSV proviral DNA in this study. Two separate subclones of v-fes in pBR322 plasmid were provided by Dr. Genoveffa Franchini (National Cancer Institute, Bethesda, MD) (Figure 1). One of these subclones, referred to as \( S_L \), included the left (5'-3') Pst I restriction fragment of the v-fes gene of ST-FeSV, and the other, referred to as \( S_R \), included the right
Pst I restriction fragment of the v-fes gene. Each fragment (approximately 0.5 kilobase pairs long) was separately subcloned into the ampicillin-resistance gene of the plasmid vector, pBR322 (Franchini et al., 1981). Both subclones were used to detect v-fes homology in this study.

The second approach involved four basic steps:

1) Human skin fibroblasts (D-550) were grown in vitro and treated with known chemical carcinogens or noncarcinogens 2 hrs before or after infection with ST-FeSV. The chemicals tested involved several hydrazines because this project was funded by the U.S. Department of the Air Force with specific interest in evaluating carcinogenic chemicals used in their worldwide operations. Hydrazine is a widely used propellant in rocketry (Wald et al., 1984).

2) Nuclear and cytoplasmic DNA fractions were isolated and purified from chemically-treated and/or ST-FeSV-infected D-550's and non-infected controls.

3) Isolated v-fes DNA was radiolabelled with $^{32}$P by nick-translation and used as a probe in nucleic acid hybridization studies.

4) Dot-blot hybridization of $^{32}$P-labelled v-fes DNA to cellular DNA was used to detect unintegrated ST-FeSV provirus in the cytoplasm and to quantitate the genome copy numbers of v-fes present in nuclear DNA.

Very little quantitation of genome integration by transforming viruses has been done in other host systems treated with chemical carcinogens. Treatment of Chinese hamster embryo cells with the
carcinogen 4-nitroquinoline 1-oxide (4-NQO) 2 hrs before infection with simian virus 40 (SV40), resulted in 2-fold enhancement of the level of SV40 DNA integrated into cellular DNA as compared to controls (Hirai et al., 1974). 4-NQO produced DNA strand scission which may have facilitated integration. In addition, it was suggested that 4-NQO might have made nonpermissive cells permissive for viral replication, increasing the number of molecules of SV40 DNA available for integration, or it might have modified the uptake of SV40 into cell nuclei by altering membrane permeability.

In contrast to this system, a number of cell lines, when pretreated with various polycyclic aromatic hydrocarbons before infection with adenovirus 5, showed enhanced viral transformation but no alteration in viral DNA synthesis or integration (Dorsch-Häsler et al., 1980). DiPaolo et al. (1977) also reported that chemical treatment did not alter simian adenovirus 7 (SA-7) genome integration in transformed colonies of Chinese hamster embryo cells. SA-7 transformation was enhanced by methyl methane sulfonate and NiSO₄, as was the total adenovirus genome content. However, increased incorporation of SA-7 DNA shortly after treatment was believed to be a result of increased uptake of viral DNA on a per-culture rather than a per-cell basis (DiPaolo et al., 1977).

The effects of chemicals on genome integration by an RNA tumor virus were also investigated. Tsuruo and Baluda (1977) determined that the viral genome equivalents of Rous sarcoma virus (RSV) integrated per cell (chicken embryonic fibroblast) treated with UV-radiation or 4-NQO at various times before or after infection did
not differ from that in untreated, infected cells. The formation and repair of random single strand breaks and gaps in cellular DNA did not facilitate integration of proviral DNA and integration was believed to be restricted.

The results from these studies demonstrated that chemicals selectively affected genome integration, depending upon the chemical's mode of action and the particular viral-host system under investigation. The purpose of this study was to determine whether select carcinogens altered the number of genome copies of ST-FeSV (specifically v-fes) which integrated into human fibroblast cells chemically treated 2 hrs before or 2 hrs after virus infection when compared to ST-FeSV-infected cells only.

Chemicals might affect a number of factors in addition to proviral integration which could lead to inhibition of transformation by ST-FeSV. They might interfere with proviral synthesis and/or inhibit transport of proviral DNA into the nucleus. Chemicals might also cause repression of integrated proviral gene functions or alter the target sites for viral gene products. Any or all of these mechanisms could cause reduced transformation frequency.

This study was valuable in understanding the co-carcinogenic interaction of select chemicals and ST-FeSV in the oncogenic process in human cells by:

1) providing information that different types of chemicals may influence retroviral integration and/or the transforming capacity of ST-FeSV in different ways,
2) providing greater understanding of the capability of certain chemicals to affect the oncogenic process differently depending on their addition previous to or after infection with ST-FeSV,

3) providing evidence that noncarcinogens as well as carcinogenic chemicals may affect ST-FeSV integration in human skin fibroblasts, and

4) providing evidence the carcinogenic chemicals do not appear to influence migration of ST-FeSV proviral DNA from the cytoplasm into the nucleus of infected human cells.
Figure 1.

Restriction endonuclease map of ST-FeSV v-fes region and cat cellular DNA sequences with homology to v-fes. The top line represents the ST-FeSV provirus from 5' to 3' with respect to viral RNA and shows the two Pst I fragments (S_L and S_R) which were separately subcloned into pBR322. Together they represent about 80% of the total v-fes gene. The middle and bottom line demonstrate the c-fes region of cat cellular DNA and the specific sites which hybridize to S_L and S_R. The v-fes sequences appear to have arisen from at least four noncontiguous sequences in cat cellular DNA which are separated by intervening sequences. (Courtesy of Dr. G. Franchini, National Cancer Institute, Bethesda, MD; Franchini et al., 1981).
Figure 1

FeSV

5'  KpnI  Sall  SacI

PstI  PstI  PstI  PstI

3'

1 Kb

S_L  S_R

c-fes

KpnI  PstI  BamHI  SacI  PstI

1 Kb

c-fes and flanking sequences
Figure 2.

Restriction endonuclease map of the human c-fes locus. The slashed bars indicate the regions which are homologous to $S_L$ and the solid bar represents the region which is homologous to the $S_R$ subclone of v-fes. The human c-fes locus is similar to the cat c-fes locus in that both consist of coding and intervening sequences. The human c-fes locus spans a region of 3.4 kilobases and contains 1.4 kilobases of DNA homologous to the viral onc sequence interspersed with three intervening sequences. (Courtesy of Dr. G. Franchini, National Cancer Institute, Bethesda, MD; Franchini et al., 1982.)
Figure 2

Human c-fes Locus

1 Kb

5' EcoRI BglII KpnI PstI HindIII BamHI SacI PstI KpnI PstI SacI BamHI KpnI PstI SacI PstI 3'

SacI KpnI PstI SacI
Initial interest in the interaction between viruses and chemicals began in the late 1930's when the pathology of papillomas induced by methylcholanthrene (MCA) and Shope papilloma virus was studied by Rous and Kidd (1938). They induced benign skin tumors in rabbits with tar or MCA. After infection with Shope papilloma virus, these benign growths rapidly became carcinomas. This work was followed by similar studies of malignant conversion of skin papillomas. Rogers and Rous (1951) inoculated rabbit skin with Shope papilloma virus after having made it previously hyperplastic with turpentine. Subsequent application of 20-methylcholanthrene (20-MCA) or 9,10-dimethyl-1,2-benzanthracene resulted in carcinomatous papilloma growths. After Southam et al. (1969) treated the skin of mice with MCA and infected them concurrently with Herpes simplex, West Nile, or Bunyamwera viruses, they saw a significant increase in the incidence of skin papillomas above that produced by MCA alone. Similar studies by Tanaka and Southam (1962, 1965) in the early 1960's explored the interactions of West Nile or Herpes simplex virus and chemical carcinogens in their combined capacity to induce malignant papillomas in mice.

Interest in the oncogenic RNA and DNA viruses expanded in the early 1970's. Investigations of their interactions with chemicals
generally showed alterations in transformation. Chemicals were shown also to be valuable for inducing the expression of endogenous virus genomes not normally expressed in the host.

Because of the broad diversity of chemical-viral interaction studies which were conducted in the 1970's and up to the current time, these investigations are categorized broadly, based on virus or viral gene expression affected by chemicals, mode of action of various chemical groups, or critical factors involved in the interaction of viruses and chemicals.

Chemical Interactions With Oncogenic RNA Viruses

Most of the investigations of chemical intervention in RNA virus expression were conducted with the murine retroviruses, but RNA viruses of avian and feline origin were studied as well as recently discovered type C viruses from human sources.

Rat embryo cells were transformed by the combined action of 3-MCA and Rauscher murine leukemia virus (MuLV) in a study done by Price et al. (1971). Cells treated with either virus or chemical alone were not transformed. Two different morphological types of transformation were observed, depending on the dose of 3-MCA. They postulated that the oncogenic effects of Rauscher MuLV were controlled by repressors which could be derepressed in different ways depending on the dose of chemical used.

Several agents were used to induce murine type C retroviruses. The most effective were the halogenated pyrimidines, including 5-iododeoxyuridine (I\text{UDR}) and 5-bromodeoxyuridine (B\text{UDR}). Chemicals
which failed to induce MuLV included cytosine arabinoside, 8-azaguanine, 6-mercaptopurine, 6-azathymine, 6-azaanuridine, dibutyryl cyclic AMP, hydroxylamine, uracil mustard, ethyl methane sulfonate (EMS), cyclophosphamide, and 5-fluorodeoxyuridine. Some degree of MuLV activation was observed with mitomycin C, 5-iododeoxycytidine, 3-MCA, and 7,12-dimethylbenz[a]anthracene (7,12-DMBA) (Hirsch and Black, 1974). Imai et al. (1982) were unable to alter mouse mammary tumor virus (MMTV) and MuLV antigen expression in any of several organ systems in mice after treatment with urethane which induced a higher incidence of mammary tumors.

A cell culture model system was developed by Howard et al. (1983) to study the interaction of the carcinogen, 7,12-DMBA and MMTV with presumptive murine mammary epithelial cells. Exposure of these cells to MMTV followed by 7,12-DMBA resulted in transformation as indicated by increased tumorigenicity in nude mice, increased ability of cells to grow in semisolid media, increased multinucleation after exposure to cytochalasin B, enhanced ability to proliferate in low Ca^{++} (0.01 mM) medium, and morphological changes. This apparent co-carcinogenic interaction between 7,12-DMBA and MMTV occurred only when 7,12-DMBA treatment followed MMTV infection and not in the presence of either agent alone. A similar temporal relationship was observed when rat and mouse embryo cells were transformed by combination treatment with diethylnitrosamine and MuLV (Freeman et al., 1970), or 3-MCA and Rauscher MuLV as mentioned previously (Price et al., 1971).

The effects of chemicals on MuLV expression were studied by Majone et al. (1983). They obtained T lymphocytes from spleens of
BALB/Mo mice which carry the Moloney MuLV endogenously and of control BALB/c mice. After treatment in vitro with hexavalent chromium or mitomycin C (MMC) in the presence of BUdR, these clastogens caused a marked increase in sister chromatid exchanges (SCE) in both BALB/c and BALB/Mo lymphocytes. An enhanced number of SCE's was seen in BALB/Mo lymphocytes indicating that Moloney MuLV and chemical carcinogens were acting synergistically to induce SCE. The synergistic effect was most marked after MMC treatment and SCE's were more effectively produced by the simultaneous action of carcinogen and BUdR. The combined action of Moloney MuLV and chemicals was thought to enhance SCE's by interfering with certain enzyme functions such as those of DNA topoisomerases. Since the DNA replicons in BALB/Mo lymphocytes were found to be larger than those in BALB/c, more topoisomerase activity was apparently required to unravel the DNA and enhance the opportunity for error.

Chemicals were also involved in the recent isolation of a new acute transforming murine type C retrovirus (Rapp et al., 1983). This virus was isolated from mice after inoculation with a stock of MuLV which was obtained after IUdR activation of C3H/10T1/2 mouse cells, transformed by 3-MCA. This virus, designated 3611-MSV is an apparent recombinant between a murine type C virus (Moloney MuLV p12 specificity) and as yet undefined transformation-specific sequences of probable mouse cellular origin.

The effects of chemicals on retroviruses of other than murine origin were investigated. Weiss et al. (1971) induced avian leukosis viruses from both group-specific (gs) antigen positive and negative,
normal chicken cells after treatment with ionizing radiations or chemical mutagens and carcinogens. Chemical treatments included 20-MCA, 4-NQO, urethane, and MMC. Conversion of gs⁻ to gs⁺ cells without simultaneous virus release indicated that viral induction by carcinogens was not a one-step process.

Another avian retrovirus, the Schmidt-Ruppin strain of Rous sarcoma virus (RSV), was recovered from an infected, transformed hamster cell line after treatment with benzo[a]pyrene (BP), 3-MCA, 4-NQO, 5-azacytidine, or dimethyl sulfoxide (DMSO) (Altanerová, 1972). The mechanism for virus induction was unknown. Altanerová (1979) also observed various degrees of rescuability of RSV after infection of chemically transformed, baby hamster kidney cells. Virus was rescued from cells treated with BP, 3-MCA, or 4-NQO. Very low levels of virus were obtained from cells treated with DMSO, and none was recovered from cells treated with 5-azacytidine. This difference in rescuability was not due to differences in virus penetration.

In contrast to many of the preceding studies which involved inbred animal cells in culture, the first evidence of successful in vivo, co-carcinogen-induced neoplasia in outbred animals was reported by Schaller et al. in 1979. The chemical carcinogen methylnitrosourea (MNU) was very effective in causing resistant adult cats to become susceptible to FeLV infection and to subsequently develop thymic lymphosarcomas. It was speculated that MNU-mediated immunodepression was involved in the enhanced susceptibility of cats to FeLV.
Chemicals were also recently used to induce a type C retrovirus from human teratocarcinoma cell lines. IUdR, m-dexamethasone, and DMSO were used simultaneously to induce the spontaneous synthesis of human teratocarcinoma-derived particles (Boller et al., 1983).

In summary, chemicals are apparently diverse in their mode of action and, depending on the chemical, may or may not induce murine type C or D retroviruses. Their interaction sets up unique conditions resulting in transformation under circumstances in which neither agent is capable of causing transformation by itself. The temporal relationship of virus infection and chemical treatment may be critical in inducing transformation. Various other consequences may result from the co-carcinogenic process such as induction of SCE or other genetic abnormalities. Type C viral induction by chemicals may be a multistep process in the avian system. Rescuability of avian retroviruses from mammalian cells or human type C viruses from human cells may be differentially affected by chemicals. Chemicals may also have various effects on immune functions which may influence virus expression in vivo.

**Chemical Interactions With Oncogenic DNA Viruses**

The effects of chemicals on or in combination with the oncogenic DNA viruses was investigated by a number of workers. Viruses studied included Simian Virus 40 (SV40), polyoma, adenovirus, and Epstein-Barr virus (EBV). Lavi et al. (1983) found that a variety of carcinogens were capable of inducing the amplification of the integrated SV40 genome and flanking cellular sequences in transformed Chinese hamster embryo cells. Successive treatment with EMS and aflatoxin B₁
(AB_1) caused the greatest amplification. Lesser amounts were observed after cells were exposed to 7,12-DMEB and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). The kinetics of amplification were identical in both chromosomal and extrachromosomal DNA. Amplification appeared to be a transient process which was detected shortly after exposure to the carcinogens (as early as one day) and which disappeared after a few days. It was speculated that carcinogens might block a large fraction of the cells in S phase. Lavi et al. (1983) further investigated the possibility that genes associated with the early S phase replicons were amplified, since SV40 sequences replicated early in S phase. Similarly, a ten-fold amplification in integrated polyoma virus sequences and flanking cellular sequences up to two kilobases distant from the polyoma virus genome, were observed after treatment of polyoma transformed rat cells with MMC (Baran et al., 1983).

In another study with SV40, 4-NQO enhanced the frequency of SV40-induced transformation of Chinese hamster embryo cells (Diamond et al., 1974). Treatment with 0.4 μg/ml of 4-NQO for 2 hr at 2 or 7 hr preinfection produced a 2- to 10-fold increase in transformation, while treatment up to 8 hr post-infection enhanced transformation by 2- to 3-fold. SCE formation and infectious virus were produced in SV40-transformed hamster kidney cells after exposure to MMC or EMS (Kaplan et al., 1978). MMC induced SCE at much lower concentrations than did EMS. Virus production paralleled SCE frequency in its response to increasing doses of MMC or EMS. The inclusion of BUDR increased the absolute yield of SV40 by 2- to 5-fold but had little
effect on the dose-dependent response to alkylating agents. Mechanisms of virus induction and SCE formation may involve DNA repair with some common enzymatic features. Kaplan et al. (1978) speculated that SCE might be associated with an error-prone repair process because of the observed correlation between SCE's, mutagens, and carcinogens. MMC also caused a 700- to 3,000-fold increase in virus yield in inducible cell lines transformed by polyoma virus (Fogel and Sachs, 1970).

Chemicals have caused enhancement of adenovirus transformation in vitro. Significant increases in the number of virus-transformed cell foci resulted when adenovirus 12-infected hamster embryo cells were treated with MNNG, hydroxylamine, or caffeine (Ledinko and Evans, 1973). Hamster embryo cells were treated with BUdR, IUdR, 5-bromo-deoxycytidine, and dibenz[a,h]anthracene prior to infection with oncogenic, adenovirus 7 (Casto, 1973). The resulting 3- to 8-fold enhancement of transformed foci relative to untreated cells was not caused by selection of transformation-sensitive cells by the chemical treatment. Casto (1973) postulated that chemicals might enhance adenovirus induced-transformation by several mechanisms:

1) chemical agents might depress cellular systems which normally degrade the viral DNA in the host cell,

2) interaction with certain physical or chemical agents could result in increased uptake of virus by the cell, or

3) disruption of the integrity of the cell DNA by the chemical agents might provide additional sites for integration of the
viral genome or increase its susceptibility to transformation by the virus.

Various chemicals, primarily halogenated pyrimidines, were used to induce the production of EBV particles or antigens. BUdR at 30 μg/ml was found to induce EBV particles in the human lymphoblastoid cell line, Raji, several weeks after removal of the drug (Hampar et al., 1972). Similarly, Gerber (1972) found that EBV synthesis was activated in up to 8% of various human lymphoid cell lines treated with BUdR at 5-25 μg/ml. Inhibitors of DNA synthesis, including 1-β-D-arabino-furanosylcytosine (Ara-c) and hydroxyurea (HU) were used to study the programming of EBV activation by BUdR and IUdR. Ara-c or HU, added at the time of removal of IUdR, prevented completion of the S phase in nonproducer Raji cells and EB-3 producer cells, without any apparent effects on the time of synthesis of Early antigen (EA) (Hampar et al., 1974a). Ara-c, HU, and excess thymidine were all found to enhance virus activation in EB-3 cells and in BUdR-sensitized Raji cells (Hampar et al., 1974b). The critical period for EBV activation by inhibitors of DNA synthesis or halogenated pyrimidines was the S-1 phase. The sequence of EBV activation by cycloheximide or puromycin (inhibitors of protein synthesis) was similar to that for halogenated pyrimidines in that treatment was necessary during the S-1 phase of the cell cycle (Hampar et al., 1976).

The latent state of the EBV genome is believed to require a tight association of the cellular DNA and the episomal EBV DNA. Temporary dissociation probably occurs during replication and activation.
Incorporation of halogenated pyrimidines is believed to alter the binding capacity of the cell or viral DNA for a DNA binding protein which initiates activation. The mechanism by which cycloheximide activates EBV expression is not readily apparent. A certain period of DNA synthesis after the addition of cycloheximide is required. By slowing down the length of time for DNA synthesis, cycloheximide may induce a state of "fragility" of the cell and/or viral DNAs which reduces the probability of their reassociating to maintain the latent virus state (Hampar et al., 1976).

MNNG induced transformation of human umbilical cord lymphocytes when added after EBV infection, whereas methyl methane sulfonate (MMS) did not (Henderson and Ribecky, 1980). Differences in reactivity to MMS and MNNG were not explained by DNA repair in that each of these chemicals induced similar amounts of repair at equitoxic doses.

In summary, chemicals may cause amplification of viral DNA sequences in transformed host cells. They have also enhanced the frequency of transformation, SCE formation, and recovery of infectious virus from cells infected with oncogenic DNA viruses. DNA repair processes and the cell cycle may play an important role in chemical amplification and induction of DNA viruses.

Importance of Time of Virus Infection Relative to Chemical Treatment

Time of chemical treatment relative to virus infection may play an important role in the transformation process. When hamster embryo cells were treated with BP, 3-MCA, 7,12-DMBA, dibenz[a,h]anthracene, or dibenz[a,c]anthracene 18 hr prior to infection with simian adenovirus 7 (SA-7), the frequency of viral transformation was enhanced.
If treatment was delayed until 5 hr post-infection, however, transformation was inhibited (Casto et al., 1973). Pretreatment of cells with chemicals appeared to increase their sensitivity to SA-7-induced transformation. Because noncarcinogenic polycyclic aromatic hydrocarbons such as phenanthrene, pyrene, and perylene failed to stimulate transformation by SA-7, Casto et al. (1973) speculated that agents capable of disrupting the stability of cellular DNA increased the susceptibility of hamster embryo cells to transformation by SA-7.

An analogous situation occurred when human fibroblast cells were treated with naphthylamine-2, MNNG, 2-acetylaminofluorene (2-AAF), BP, AB₁, propane sultone (PS), β-propiolactone, 4-NQO, MMS, or diethyl nitrosamine (DEN) prior to infection with SV40. Transformation was enhanced relative to virus-infected control cells (Milo et al., 1978). With the exception of 4-NQO, MMS, MNNG, and DEN which caused inhibition, no change in transformation occurred when the chemicals were applied after virus infection. Treatment of cells in vitro with pyrene, anthracene, or weak or non-carcinogens, either before or after infection with SA-7 or SV40 did not alter virus-induced transformation (Casto et al., 1973; Milo et al., 1978). Since MNNG, BP, and 2-AAF induced breaks in cellular DNA and weak or non-carcinogens did not, Milo et al. (1978) supposed that damaged DNA provided additional sites for viral integration which was a prerequisite for SV40-induced transformation. However, DNA damage most likely was not the only cause for enhanced transformation, in that MNNG, 2-AAF, and BP enhanced virus transformation when added 24 hr
pre-infection whereas all detectable repair synthesis ceased after 10 hr.

In summary, certain chemicals affect virus expression differently depending upon when they are added in relation to virus infection. In general, pre-treatment with chemicals appeared to be more effective in inducing SV40 and SA-7 transformation. Chemical modification of viral or cellular DNA may play a role in time-dependent transformation processes.

Effects of Chemicals on Virus Integration

The effects of chemicals on the integration of potentially oncogenic viral genomes has been examined in both DNA and RNA virus systems. Four days after treatment with 100 µg/ml MMS or 3 days after treatment with 400 µg/ml of NiSO₄, DiPaolo et al. (1977) observed a 12-fold and a 4-fold increase, respectively, in viral genome content relative to Syrian hamster cells infected with SA-7 alone. When virus was added immediately after MMS treatment, SA-7 genome content increased by 4-5 times during the first 24 hr, in the absence of scheduled DNA synthesis. When virus was added 24 hr after MMS, no increase occurred. Insertion of the SA-7 genome was apparently random among all chromosomes and nuclei. It was not determined whether increased incorporation of SA-7 DNA was due to an increased uptake on a per culture or per cell basis.

Dorsch-Häslcr et al. (1980) compared the state of integration of viral DNA in transformed clones isolated from secondary rat embryo cells infected with adenovirus 5 mutant, H5ts125, with or without previous exposure of the cultures to either of the initiating
carcinogens, BP or 7,12-DMBA. Neither BP nor DMBA altered the extent or pattern of adenovirus DNA integration. It was found that even when multiple copies of viral DNA were present, they existed in intact linear sequences, but not as concatemers. The viral DNA did not appear to integrate in a unique site in the host cell genome.

As previously mentioned, Chinese hamster embryo cells treated with 4-NQO 2 hr prior to SV40 infection demonstrated an increase in frequency of cell transformation. The quantity of SV40 DNA integrated into cellular DNA also increased but it could not be determined whether there were more SV40 genomes integrated per cell or more cells with integrated genomes (Hirai et al., 1974). When chicken embryonic fibroblasts were treated with UV irradiation or 4-NQO at different time intervals before or after infection with Rous sarcoma virus (RSV) (Schmidt-Ruppin strain), the copy number of viral genome equivalents integrated per cell did not differ from the copy number in untreated but infected cells (Tsuruo and Baluda, 1977).

In summary, it appears that carcinogens may have variable effects on viral genome integration. Depending upon time of treatment, carcinogens enhanced the SA-7 genome content of infected cells, but had no effect on the extent or pattern of adenovirus 5 DNA integration. Chemicals increased the quantity of SV40 DNA in infected cells, but neither pre- nor post-treatment affected RSV proviral integration.

Importance of the Cell Cycle in Chemically-Induced Virus Activation

The importance of the cell cycle and S-1 phase for Epstein-Barr virus activation by halogenated pyrimidines and inhibitors of DNA and
protein synthesis has already been cited. Induction of endogenous, xenotropic type C virus from Kirsten sarcoma virus-transformed BALB/c (K-BALB) mouse cells also depended on DNA synthesis during S phase (Suk et al., 1981). Virus induction by IUdR, cycloheximide, and histinol was inhibited by the addition of L-β-3,4-dihydroxyphenylalanine (L-dopa) methyl ester which apparently suppressed the progression of the cells into S phase.

Endogenous Virus Activation by Chemicals

Many different agents are capable of inducing endogenous retroviruses. Some of the agents used to induce the endogenous murine leukemia viruses have included the halogenated pyrimidines (Lowy et al., 1971; Aaronson et al., 1971; Teich et al., 1973; and Ihle et al., 1974), cycloheximide (Aaronson and Dunn, 1974; Greenberger and Aaronson, 1975), L-canavanine and other amino acid analogs (Aksamit and Long, 1977; Aksamit et al., 1977), acute X-irradiation (Tennant et al., 1976; 1977), low dose gamma irradiation (Tennant et al., 1976; Otten et al., 1976), various chemical carcinogens (Teich et al., 1973; Tennant et al., 1977), and HU and other related compounds such as carbamoyloxuurea and formamidoxime (Tennant et al., 1977; Rascati and Tennant, 1978). Most of the studies involved with virus induction from cultured mouse cells generally did not produce viruses with direct oncogenic potential. These investigations provided important insights into the regulation of cellular gene expression, however, since the viral loci are under cellular control.

Teich et al. (1971) tested various compounds for their ability to activate MuLV synthesis in AKR mouse embryo cells. IUdR and BUdR
were superior to all other compounds tested in reducing the induction time and in the production of infectious virus. 5-Iododeoxycytidine induced virus at lower amounts than BUdR and IUdR, probably by intracellular conversion to IUdR after deamination. 3-MCA and 7,12-DMBA induced MuLV synthesis in some experiments. The concentration of chemical and length of treatment were both factors in the efficiency of MuLV activation. Inhibition of analog incorporation prevented activation.

The molecular mechanism for induction of MuLV by BUdR and IUdR has not been defined. Both incorporated and unincorporated analogs interfere with cellular metabolism, but in different ways. Unincorporated BUdR and IUdR inhibit the synthesis of thymidine nucleotides by competitive inhibition for thymidine kinase. At sufficiently high concentrations, BUdR and IUdR also decrease the total synthesis of DNA nucleotides by feedback inhibition. When these analogs are incorporated into DNA they function in increasing the mutation rate, inducing single strand and chromosomal breaks, increasing the degradation of DNA, decreasing enzyme synthesis, and in reducing the rate of DNA synthesis. It was difficult for Teich et al. (1971) to determine which of these might be critical in MuLV activation. They also found that the mutagenic and carcinogenic alkylating agents, EMS and MMS, were unable to activate MuLV synthesis. MMC, however, which is also a mutagenic alkylating agent, induced low amounts of virus.

HU induced endogenous MuLV at low frequency and in a manner similar to low-dose-rate gamma irradiation and acute X-irradiation (Rascati and Tennant, 1979). A requirement for induction of MuLV by
HU was that the DNA must undergo semiconservative replication shortly after HU treatment. Repair synthesis followed HU treatment. If replicative synthesis preceded repair, virus was induced, but if repair occurred prior to DNA replication, virus induction was inhibited. Halogenated pyrimidines had a synergistic stimulatory effect on HU-mediated induction when added late in the HU treatment period. They were presumably incorporated into cellular DNA during repair replication. The type of DNA lesion caused by HU was not determined. Rascati and Tennant (1979) suggested that HU might cause single strand breaks at very low frequency which were quickly repaired. Alternatively, a chemical modification of the DNA might have occurred or HU might have caused the release of a metabolic or an oxidative breakdown product. A repairable lesion in the DNA was thought most likely to be involved. Subsequent steps might have included excision of the viral genome and reintegration at some site not subject to normal control processes, involved with virus repression. An alternative hypothesis was that the modified DNA and/or the IUdR-substituted DNA might have altered binding affinity for a repressor-like molecule which controls virus expression. Further support for this theory came from the ability of cycloheximide, puromycin, and amino acid analogs to induce virus expression since these agents would inhibit the synthesis of a functional repressor.

In a comparison of twelve different mouse cell strains for their capacity to induce endogenous retrovirus proteins following exposure to IUdR, Tennant et al. (1982) found that the frequency of induction was highest in AKR cells. It was also higher in all inducible mouse
cells than the mutation frequency commonly observed with other mutagens. BP was also able to induce endogenous virus expression in AKR cells. This induction required metabolism of BP by cellular monooxygenase enzymes to the more reactive 7,8-dihydroxybenzo[a]-pyrene and 7,8-dihydroxy-9,10-epoxybenzo[a]pyrene. Further studies were done on the induction capacity of substituted nucleosides. Deoxycytidine, when substituted at the 5 position with a nonpolar methyl group did not induce retrovirus expression. Substitution of nucleotides with nonpolar groups apparently did not allow for sufficient perturbation of the DNA structure to cause virus induction, particularly if the nonpolar group was small and nonbulky. Alkylating agents such as EMS, N-nitroso-N-ethylurea, MMS, and MNNG also cause nonpolar, nonbulky modification of the DNA and were not capable of inducing murine retrovirus expression. Agents such as BP, 2-AAF, AB₁, and possibly isopropyl methane sulfonate cause bulkier substitutions and were able to induce virus expression. Instability of the induced state suggested that induction could involve mechanisms other than point mutation.

Results obtained with 5-azacytidine (AZA) provided the most convincing evidence for a nonmutagenic effect in MuLV induction (Niwa and Sugahara, 1981; Tennant et al., 1982). AZA induced virus protein expression in greater than 95 per cent of exposed cells which far exceeded the mutation frequency for other specific loci. Although mutagenic, AZA also is known to result in undermethylation of newly synthesized DNA, since the nitrogen substituted 5 position of AZA is
incapable of being methylated. Tennant et al. (1982) concluded that hypomethylation could account for the transcriptional activation of endogenous MuLV. Hence, some mutagens might act on cellular regulatory functions. It was also suggested that 2-AAF might turn on endogenous murine retrovirus expression in AKR mouse embryo fibroblasts by its ability to inhibit methylation of DNA (Rascati and McNeely, 1983).

Caffeine was studied for its effects on the expression of murine endogenous viruses in two different mouse cell lines (Niwa and Sugahara, 1981). The K-BALB cell line harbors both a xenotropic and an ecotropic virus and the AKR2B cell line carries an ecotropic MuLV. When these cells were treated with caffeine after IUdR induction, there was almost no effect on normal IUdR-induced virus expression from K-BALB cells. In contrast, caffeine exhibited a strong inhibitory effect for virus expression in the AKR2B cell system. The pathway for endogenous MuLV induction appeared to be different for these two cell lines.

Syn-carcinogenic effects between MNU and endogenous MuLV were observed by Fey (1981). When activated endogenous MuLV, isolated from MNU-induced leukemias, was inoculated into mice before suboptimal doses of MNU were given, oncogenesis was enhanced.

A variety of both organic and inorganic, mutagenic selenium compounds were investigated for their ability to induce endogenous retrovirus expression in cultured AKR mouse embryo fibroblasts (Rascati, 1983). Only selenomethionine was capable of inducing virus expression. The mechanism for virus induction by selenomethionine
was not determined although mutation did not appear to be the cause. One interesting explanation offered was that selenomethionine might be unable to function as a methyl donor or might somehow interfere with the intracellular production of 5-adenosyl-methionine. This would interfere with the methylation process and result in under-methylated DNA and subsequent induction of endogenous virus expression. This theory also explained the need for actively growing cells in which DNA is being synthesized, since methylation occurs after DNA replication.

Actinomycin D and HU have been used to gain information about the mechanism of induction of endogenous, xenotropic type C virus from Al-2 cells (derived from BALB/c mouse) by UV-irradiated Herpes simplex virus (Brewer and Helman, 1980). HU decreased virus induction, indicating a requirement for active DNA synthesis. Virus induction was even more sensitive to inhibition by actinomycin D. The actinomycin D sensitivity of the UV-HSV induction in this study was similar to that reported for cycloheximide induction of type C virus (Greenberger and Aaronson, 1975). Because both HU and actinomycin D were inhibitory, it was probable that both DNA and RNA synthesis were involved in a complex series of events leading to the induction of xenotropic type C virus by UV-irradiated HSV.

Endogenous retroviruses in the avian system were also activated by chemicals. Chicken cells contain one or more complete or nearly complete copies of genetically transmitted endogenous retroviruses. Most chickens contain one such genetic locus referred to as ev-1, which generally remains silent and unexpressed. Transient exposure
of cells to AZA resulted in transcriptional activation of the ev-1 locus. Undermethylation of at least part of ev-1 resulted in DNase sensitivity, transcription, and synthesis of viral proteins (Groudine et al., 1981). The chlorinated hydrocarbon, DDT, and its metabolites induced endogenous avian retrovirus gene expression in chick embryo cells (Pearson et al., 1981). At subtoxic levels, these chemicals caused the induction of virus particles and reverse transcriptase activity in about 35-40 per cent of exposed cells.

In summary, a wide range of agents are capable of activating endogenous murine retroviruses. In particular, the halogenated pyrimidines and certain polycyclic aromatic hydrocarbons are very effective and have been most studied. Chemical effects on cellular DNA synthesis and methylation of proviral DNA may be important in RNA type C virus activation.

Effects of Tumor Promoters on Oncogenic RNA and DNA Viruses

Since the late 1970's, the interaction between tumor promoting chemicals and the oncogenic RNA and DNA viruses has received serious attention. One of the largest classes of promoting chemicals is the diterpene esters, which includes 12-0-tetradecanoyl-phorbol-13-acetate (TPA). Though not significantly carcinogenic when tested alone, they decrease the threshold dose of initiating carcinogens and reduce the latency period for tumor appearance.

TPA caused a 5- to 20-fold stimulation of primate retrovirus synthesis in persistently infected human embryonic E1, human embryonic kidney, and Tu 197 cells, which are all virus producers (Wunderlich et al., 1981; 1983). Cells were infected with type C or
type D viruses including Baboon endogenous type C virus, Simian sarcoma virus, Mason-Pfizer virus of Rhesus monkeys and PMFV, which is a type D virus from human cells. Enhanced virus expression was paralleled by striking morphological alterations of the cells. TPA-mediated stimulation of retrovirus synthesis was transitory. When virus-producing cells were cultured continuously in the presence of TPA, maximal stimulation of virus production occurred on day 2 or 3. Thereafter, cells lost their ability to respond to freshly added TPA with enhanced virus production and returned to basal levels.

Results with another tumor promoter, teleocidin, an indole alkaloid from *Streptomyces mediocidicus*, were nearly identical biologically, despite an entirely different chemical structure compared to TPA.

When Arya (1980) treated a line of C3H mouse mammary tumor-derived cells, which produce MMTV, with TPA, virus production was enhanced in the absence of significant effects on cell growth. A maximum increase in virus production of 10- to 12-fold was obtained with a TPA concentration of 10 to 20 ng per ml. When dexamethasone (synthetic glucocorticoid) was added in addition to TPA, the combined effect resulted in a 100- to 200-fold enhancement of MMTV production. Virus enhancement was specific for TPA. The non-tumor promoter, phorbol, did not stimulate virus production up to a concentration of 100 ng/ml. It was not determined whether TPA directly affected some activity specific for virus synthesis or whether its effect was mediated through some cellular function.

The recently discovered tumor promoter, dihydroteleocidin B, in addition to the tumor-promoting phorbol esters, phorbol-12, 13-dide-
canoate, and TPA all enhanced the production of Moloney MuLV in a mouse fibroblast cell line, C3H2K, which is persistently infected with Moloney MuLV (Hoshino et al., 1983). Treatment with dihydroteleocidin B for 1-7 days at 10 or 100 ng/ml resulted in a 4- to 5-fold enhancement of infectious Moloney MuLV relative to control cells. These tumor promoters were not capable of inducing endogenous MuLV in C3H2K cells or ecotropic and xenotropic MuLV in K-BALB cells, at concentrations up to 1,000 ng/ml.

TPA caused a significant acceleration of early gene expression in HeLa cells infected with adenovirus type 5 (Carter et al., 1984). Its action resembled that of the adenovirus 5-Ela regulatory product, which if absent in mutants causes a considerable delay in early gene expression. TPA did not alter the kinetics of viral uptake. Both TPA and phorbol didecanoate, which are strong tumor promoters, enhanced the incorporation of \(^{3}H\)-labelled UTP into RNA after virus infection. No enhancement occurred with phorbol and 4-alpha-phorbol-didecanoate which are both inactive as tumor promoters on mouse skin. TPA exerted its effects on adenovirus transcription only when intact cells were treated. No effects were observed when nuclei were separately exposed to TPA. In the adenovirus system, TPA apparently decreased the latent period before initiation by RNA polymerase at early viral promoters. This acceleration was not dependent upon concurrent protein synthesis.

TPA markedly enhanced the cloning efficiency and tumorigenic potential of cells transformed by HSV type 2 (Kucera et al., 1983). The effects were irreversible since both enhancement of cloning
efficiency and tumorigenic potential were measured after removal of TPA. Enhanced cloning efficiency was observed after transient exposure to TPA in both initiated nontumorigenic and weakly tumorigenic cells. Nontumorigenic cells required prolonged exposure to TPA (up to 20 serial passages) before tumorigenic potential was enhanced, while weakly tumorigenic cells showed enhancement of tumorigenic potential after 48 hr of exposure to TPA. This supported the idea of tumor promotion being a multistep process.

TPA induced replication of EBV DNA in the virus-producing, human lymphoblastoid cell line, P3HR-1, but not in the nonproducer cell line, Raji. There was a 6-fold increase in the number of EBV genome copies per P3HR-1 cell and a similar increase in the percentage of cells synthesizing viral capsid antigen. The optimal TPA concentration for the induction of EBV DNA replication was 10 ng/ml. In situ cytohybridization showed that most of the TPA-treated cell population participated in the virus-productive cycle (Lin et al., 1979). Yamamoto and zur Hausen (1979) also found that TPA enhanced the transformation of human cord blood lymphocytes by EBV. Transformed colonies appeared earlier and were larger in cultures maintained with TPA. No differences were observed in the morphology of TPA-treated and untreated cultures.

The efficiency of TPA in inducing EBV and other oncogenic herpesviruses such as Herpesvirus papio and Herpesvirus saimiri was further verified by the work of zur Hausen et al. (1978). They treated two EBV producer lines, P3HR-1 and B95-8, and two nonproducer lines, Raji and NC37 with TPA. A dramatic increase in antigen positive cells and
particle synthesis occurred in the producer lines. TPA was used to
treat 13 additional lymphoblastoid lines transformed by oncogenic
herpesviruses. The frequency of antigen induction varied, but some
increase in EA or Viral Capsid antigen (VCA) synthesis occurred in
every EBV-transformed cell line tested. TPA was highly efficient in
inducing EBV at \(3 \times 10^{-8}\)M. This far exceeded the effective concentra-
tion \((7 \times 10^{-5}\)M) for induction by halogenated pyrimidines such as
BUdR and IUdR, by several orders of magnitude. The mechanism of EBV
induction by TPA was unknown. TPA also appeared to have potential as
an inducing agent for SV40 and BK virus (zur Hausen et al., 1978).

TPA and RPA (12-0-retinoylphorbol-13-acetate) were each capable
of inducing the transcription of bovine papilloma virus type 1
(BPV-1) in mouse embryo fibroblasts, in which the viral genome
existed in a non-expressed episomal state after infection (Amtmann
and Sauer, 1982). One brief treatment with either chemical resulted
in the transcription of the same viral mRNA species found in BPV-1
tumors and in BPV-1 transformed cells. Once activated, the viral DNA
was stably replicated and the cells became morphologically trans-
formed. Under natural conditions in Scotland and northern England,
an apparent interaction was reported between bovine papilloma virus
and an environmental carcinogen or promoting agent, possibly from
bracken fern, resulting in alimentary malignancies in cattle (Jarrett
et al., 1978).

Seif (1980) investigated the ability of various tumor promoters
and other agents which disorganize microtubules or microfilaments, to
enhance transformation by polyoma virus. The agents examined
included griseofulvin (tumor promoter which disorganizes microtubules and the mitotic spindle and induces numerical chromosome aberrations); epidermal growth factor (tumor enhancer which disorganizes microfilaments); TPA (promoter which disorganizes microfilaments and induces abnormal chromatid exchange); melittin (suspected tumor promoter which produces effects similar to TPA); vinblastine, podophyllotoxin, colcemid, and colchicine (non-tumor promoters which disrupt microtubules and the mitotic spindle and induce aberrations in chromosome number or exchange); and cytochalasin B (non-tumor promoter which disrupts microfilaments and causes polyploidy). All of these chemicals increased the frequency of 3T3 rat cell transformation by polyoma virus, by 8- to 40-fold. None induced transformation in the absence of the virus. Griseofulvin had a dual effect on transformation. At high doses it inhibited transformation with little damage to normal cells and at low doses it increased the frequency of cell transformation. The data suggested that the enhancing activity of these chemicals might involve the mitotic spindle and its role in chromosome segregation and that tumor promotion might act similarly. A second possibility was that these agents increased the efficiency of polyoma virus integration. Seif (1980) also suggested that these agents might activate one or several cryptic oncogenes only when combined temporarily with some function of polyoma virus.

Fisher et al. (1978; 1979) studied the effects of TPA on adenovirus transformation both with and without chemical carcinogens. Pretreatment of rat embryo cells with 7,12-DMBA or BP at 18 hr prior
to infection with adenovirus 5 mutant, H5tsl25, caused a 2- to 4-fold enhancement of transformation frequency. When TPA was added to the culture medium alone at 72 hr after viral infection, transformation was enhanced 2- to 3-fold. The effect of 7,12-DMBA and BP was augmented about 2-fold when cultures were also grown in the presence of TPA. The ability of TPA to enhance adenovirus H5tsl25-transformation of rat embryo cells was dependent on their time of exposure to TPA relative to virus infection. A 2-fold increase in transformation was observed when TPA was added at 1, 3, or 7 days after infection, but by 14 to 21 days post-infection there was no enhancement. TPA also caused a striking enhancement of the growth in agar of all the transformants and it increased the saturation densities and enhanced the cloning efficiencies in liquid medium of most of the transformants (Fisher et al., 1979).

Fisher et al. (1978) speculated about mechanisms whereby promoting agents might enhance adenovirus transformation. TPA could augment virus uptake and/or integration into cellular DNA. Also, TPA might selectively enhance gene expression and growth of cells containing integrated viral DNA sequences. It appeared that TPA's effects were exerted after viral DNA was integrated.

In summary, TPA has been the most widely studied of the tumor promoters. It stimulates the synthesis of type B, C, and D retroviruses. It is also effective in inducing oncogenic herpesviruses and in enhancing their cloning efficiency and tumorigenicity. It has been used to induce many of the oncogenic DNA viruses including EBV, SV40, BK, papilloma, and polyoma, and it enhances adenovirus-5
transformation and early gene expression. Other tumor promoters in addition to TPA may activate MuLV. Their effects on the mitotic spindle and chromosome segregation may be a factor in virus induction.

**Effect of Hormones on Virus Expression**

Hormones, when present either endogenously or when added exogenously, may also influence virus expression in certain host systems. Epidermal growth factor (EGF) is a well-characterized polypeptide hormone which is a known mitogen for cells in vitro and which produces a number of effects phenotypically similar to TPA such as: induction of plasminogen activator, ornithine decarboxylase, and prostaglandin synthesis and enhancement of cell growth in liquid and agar medium (Fisher et al., 1981). On a molar basis, EGF was about ten times more potent than TPA in enhancing in vitro transformation of C3H10T
\( \text{f} \) cells infected with adenovirus H5ts125. EGF exerted its effects at about \( 10^{-9} \) to \( 10^{-10} \) M, which is in the range of its physiological concentration. Although mechanisms by which phorbol esters and EGF enhance adenovirus transformation are not known, Fisher et al. (1981) suggested that enhancement may occur via effects on Ca\(^{2+}\) metabolism. They cited studies which suggested that endogenous growth factors may play a role in maintenance of the transformed phenotype. Reduced capacity to bind EGF has been observed in cells transformed by murine or feline sarcoma viruses, adenovirus, and chemical carcinogens.

Studies indicating a hormonal effect on leukemogenesis in mice predated the discovery of the murine leukemia viruses. In the 1930's
and 1940's, studies indicated that exogenously administered estrogens increased the incidence of spontaneous lymphomas in several strains of mice. The effect of estrogens was abrogated by testosterone. More recent studies in mice demonstrated that estrogenic type compounds can induce C-type RNA virus markers such as gs antigen and reverse transcriptase activity. Compounds capable of activating these virus markers included prolactin, natural steroids such as 17β-estradiol, synthetic estrogens such as mestranol, or nonsteroidal estrogens such as diethylstilbestrol. Some of the hormonal compounds capable of enhancing the effects of IUDR on C-type particle induction have included the adrenal corticosteroids, dexamethasone, prednisolone, fluorocortisone, hydrocortisone, and corticosterone. Dexamethasone has also stimulated production of type-B MMTV (Hirsch and Black, 1974).

The C3H/St Wi strain of mice possesses the MMTV provirus endogenously, which is not normally expressed as virions. Virgin females were exposed to the chemical carcinogens, 7,12-DMBA and urethane in the presence or absence of chronic hormonal stimulation of the mammary gland by pituitary isografts. Hormonal stimulation of the glands during and after exposure to the carcinogens increased the rate of tumorigenesis, but did not favor complete expression of the endogenous MMTV genes. In positive tumors, the internal MMTV gag gene antigen, p28, was prevalent (Smith et al., 1981).

Using group-specific antisera against the C-type RNA tumor viruses, Hellman and Fowler (1971) observed gs antigen enhancement by
certain steroids after administration of estrogen and progesterone to mice. Progesterone in some cases seemed to be suppressive.

Blakeslee et al. (1975) investigated the interaction between 17β-estradiol, SV40 virus, and the DNA damaging-chemical carcinogens, MNNG and BP. Transformation by SV40 was enhanced when human foreskin fibroblast cells were treated with BP or MNNG prior to virus infection. Conversely, 17β-estradiol enhanced SV40 transformation only when the cells were treated after virus infection. Concentrations of 17β-estradiol which stimulated scheduled DNA synthesis (0.1 and 1.0 µg/ml) enhanced virus transformation independent of carcinogen treatment. When 17β-estradiol and MNNG were added together, they synergistically enhanced virus transformation.

The effects of hormones on adenovirus 12 transformation of primary hamster embryo cells were influenced by interrelationships between calcium concentration in the media, glucocorticoid activity of the steroid, and cell sex (Milo et al., 1972). Significant enhancement of transformation occurred in female cultures treated with cortisone acetate and in male cultures treated with dexamethasone. Marked inhibition of adenovirus 12 transformation occurred in both male and female cells treated with estrovarin and in male cells treated with aldosterone or progesterone. Estrone and testosterone inhibited transformation of male cells to some extent. The mechanisms of enhancement and inhibition were not determined.

Paran et al. (1973) compared the effects of various hormones on Kirsten murine sarcoma virus (MSV) induction. Dexamethasone enhanced virus production, induced by IUDR, in a transformed-nonproducer line
of BALB/3T3 fibroblasts infected with Kirsten MSV. Enhancement by
dexamethasone was completely dependent on virus induction by IUdR.
Prednisolone (natural glucocorticoid) also stimulated IUdR-induced
virus production in a similar physiological concentration range (10^{-5}
to 10^{-6} M). Dexamethasone increased reverse transcriptase activity
and focus induction by 13- to 16-fold as compared to non-treated,
induced cultures. This effect was not due to enhancement of cell
number or to IUdR incorporation into DNA. Twelve different natural
or synthetic glucogenic hormones, or combinations thereof, were
tested for their ability to induce Kirsten MSV. Stimulatory activity
was specific for the glucogenic adrenal steroids and did not occur
when estrogenic or androgenic hormones were used. Cordycepin
completely inhibited virus production, probably by inhibiting
synthesis of the poly (A) component of the viral RNA. Paran et al.
(1973) found that the glucogenic adrenal steroids which stimulated
Kirsten MSV also enhanced Rauscher MuLV production in BALB/K-3T3
cells by 3- to 5-fold.

Dehydroepiandrosterone (DHEA), which is a major adrenal secretory
product in humans, was shown to inhibit EBV-induced morphologic
transformation and stimulation of DNA synthesis in human lymphocytes
(Henderson et al., 1981). DHEA has no known biological function, but
acts as a potent inhibitor of mammalian glucose-6-phosphate dehydro­
genase (G6PDH). 16α-Bromo-epiandrosterone (Epi-Br) is a DHEA analog
which is about 60 times more potent than DHEA as an inhibitor of
G6PDH. It also is a more potent inhibitor of EBV-induced transform­
ation. Henderson et al. (1981) suggested that the inhibitory effect
of these steroids on DNA synthesis accounted for their capacity to reduce the rate of transformation of peripheral blood lymphocytes by EBV, since cellular DNA synthesis was a necessary prerequisite for morphologic transformation. These steroids may suppress DNA synthesis by inhibiting G6PDH, thereby reducing the supply of deoxyribonucleotide precursors, ribose-phosphate and NADPH, from the pentose-phosphate pathway.

In summary, many different types of hormones affect virus expression. Natural and synthetic estrogens as well as other natural steroids activate production and expression of MuLV. Hormones enhance virus induction by halogenated pyrimidines and may have synergistic effects with chemicals in enhancing transformation. The mode of action of hormones may influence their ability to affect virus transformation. For example, stimulation of Kirsten MSV and Rauscher MuLV is specific for glucogenic adrenal steroids which inhibit EBV-induced transformation.

**Effect of Chemicals on Viral Antigen Expression**

Chemicals may affect only certain select viral gene functions or products, often in the absence of particle induction. EBV-EA synthesis was stimulated by the following: the phorbol diester tumor promoter, TPA (zur Hausen et al., 1979); by a variety of treatments including culture in arginine-deficient medium (Henle and Henle, 1968); by inhibitors of protein synthesis (Gerber, 1972; Hampar et al., 1976); by halogenated pyrimidines (Hampar et al., 1972); by anti-IgM sera (Tovey et al., 1978); by n-butyric acid (Luka et al., 1979); by AZA (Ben-Sasson and Klein, 1981); by intercalating
chemicals (Yamamoto et al., 1981); and by a protein factor isolated from a wide variety of mammalian serum (Bauer et al., 1978a; 1978b; 1982; Wittman et al., 1982). No common mechanism of EBV-EA induction has yet been identified.

Treatment of Raji and other EBV genome-positive cells with indomethacin caused a marked inhibition of EA induction by TPA and other chemical inducers, including one per cent rabbit anti-human IgG antiserum, IUdR, n-butyrate, and activated serum factor (Daniel et al., 1984). The concentrations of indomethacin required to inhibit EBV-EA induction were cytostatic, again indicating that the S phase of the cell cycle might be required to render cells competent for viral antigen induction.

Yamamoto et al. (1979) investigated the interaction of retinoic acid with induction of EBV-EA by tumor promoters and other agents. Ninety per cent inhibition of EA induction occurred when cells were treated with both TPA and retinoic acid ($10^{-5}$M). Similar amounts of inhibition were observed when cells were pretreated with retinoic acid ($10^{-6}$M) for 12 hr followed by subsequent washing and addition of TPA. Induction of EA by a different tumor promoter known as Pimelea factor, P2 was also inhibited by retinoic acid. IUdR induction of EA was less strongly inhibited by retinoic acid, while anti-IgM induction of EA was clearly inhibited.

MMC, actinomycin D, methotrexate, and X-irradiation all increased Membrane antigen (MA) expression by EBV under appropriate conditions. IUdR and Ara-C treatment caused accumulation of EA in producer lines, but blocked the late production of VCA (Hirsch and Black, 1974).
When Kallin et al. (1979) exposed latently infected P3HR-1 cells to 3mM (sodium) n-butyrate, EBV-EA was induced in 70 to 90 per cent of the cells within 40 hr. In addition, after 72 hr, 50 to 80 per cent of the cells also became VCA positive. Parallel, untreated cultures contained only about 5% EA and 1 to 3% VCA-positive cells. The n-butyrate-induced viral cycle was largely abortive but nevertheless allowed for the efficient detection of early and late viral polypeptides.

In summary, the effects of chemicals on antigen synthesis by EBV have been most widely investigated. A large variety of chemicals and certain other agents induce EBV-EA with no apparent common mechanism. Some chemicals inhibit TPA-induction of EA such as indomethacin and retinoic acid. Others enhance MA or EA and VCA, while agents such as IUdR and Ara-C induce EA but block late production of VCA.

Enzyme Induction by Chemical-Viral Interactions

Elevated enzyme activities of viral or cellular origin sometimes result from combined chemical treatments and virus infection in certain host cell systems. TPA induced a DNase activity in the EBV producer cell line P3HR-1 (Tan et al., 1982). An EBV-specific DNase was also induced in EBV nonproducer Raji cells after treatment with TPA and sodium butyrate (Ooka et al., 1984). The increase in EBV DNase activity was related to the appearance of EA-positive cells and its induction did not depend on viral DNA synthesis. This DNase was thought to be part of the EBV early protein complex, appearing prior to viral DNA synthesis and related to EA production.
In studying the mechanisms by which IUdR and MMC induce SV40 replication in CV1C11 monkey kidney cells, Mezzina et al. (1982) noted alteration in cellular DNA ligase activity. It previously was observed that ligase activity increased after UV-irradiation of monkey kidney and human cells, suggesting a direct role of the ligase in DNA excision-repair by sealing the repaired strand. Treatment of cells with IUdR or MMC induced DNA damages different from the pyrimidine dimers and other lesions produced by UV-irradiation. IUdR incorporates into viral, bacterial, and mammalian DNA and inhibits its replication, while MMC, as a bifunctional alkylating agent, induces the formation of intra- and inter-strand DNA cross-links which interfere with replication. The level of ligase activity was about 2 to 3 times higher in IUdR- or MMC-treated, non-infected cells than in untreated cells. SV40 infection alone doubled the level of ligase activity. The effects of chemical treatment and virus infection were additive. DNA ligase activity was increased 3-fold in the presence of MMC and SV40, and 4-fold in the presence of IUdR and SV40. Addition of cycloheximide blocked ligase induction indicating that the enhancement observed in IUdR- or MMC-treated and virus-infected cultures probably resulted from de novo protein synthesis. Mezzina et al. (1982) postulated that the enhancement by chemicals of SV40 replication in CV1C11 cells resulted from increased activity of some cell coded enzyme(s), such as DNA ligase, involved in DNA synthesis and repair.

Roth et al. (1983) observed a 2- to 3-fold stimulation of pp60^src kinase activity (sarcoma gene product of Rous sarcoma virus) in
extracts of cells treated with 8-bromo-cyclic AMP. Stimulation of the kinase activity increased during the first 3 hr of treatment with 1mM 8-bromo-c-AMP and appeared in both the soluble and particulate fractions of the cells. It was not determined whether both the soluble form and membrane-bound form of the enzyme were activated by cyclic AMP treatment or if only the membrane form was activated and partially released into the soluble fraction. Roth et al. (1983) believed that cyclic AMP stimulated the phosphorylation of pp60src both in the soluble and particulate fractions and this increased phosphorylation caused an elevation of kinase activity. Cyclic-AMP might modulate the activity of pp60src in transformed cells.

In summary, chemicals may affect viral or cellular enzyme activities in virus-infected cells. TPA induced DNase activity in certain cells which produce EBV. IUdR and MMC cause elevated expression of cellular DNA ligase in SV40-infected cells and the tyrosine-specific protein kinase of RSV is stimulated by cyclic-AMP.

Chemical Effects on Syncytia Induction by Viruses

Chemicals also may regulate syncytia (multinuclear cells resulting from cell fusion) induction by certain viruses. Bovine leukemia virus induces syncytia in several monolayer cultures. When cultures were treated with DMSO, the number of syncytia was several times higher than in control cells and already-formed syncytia were enlarged (Itohara and Mizuno, 1984). Virus adsorption rates did not differ between DMSO-treated and control cultures. However, virus penetration was enhanced by DMSO and may have resulted in increases in syncytia formation.
Uchida and Nomura (1984) examined the effects of aliphatic amines on syncytium formation induced by HSV-1. Aliphatic amines were similar in their action to local anesthetics, tranquilizers, and ammonium salts. The number of syncytia increased when the agents were added at various times after 12 hr post-infection with HSV-1. Syncytia formation was inhibited if the agents were added 2 hr previous to their expected occurrence. The agents appeared only to prevent cell fusion and not cell-to-cell spread of infection. Inhibition was not due to toxic action by the chemicals and its mechanism was unknown.

Use of Virus Systems to Assay Chemicals for Carcinogenic or Promoting Activity

Because of the unique consistency with which certain categories of chemicals react with virus systems, a number of assays for potential carcinogens and/or tumor promoting agents have been proposed. There is a need for reliable, rapid, and reproducible mammalian assays for potentially mutagenic and oncogenic chemicals in the environment. Consistent enhancement, by chemicals, of transformation or virus expression forms the basis for most of the current assays.

Casto et al. (1977) used a transformation focus assay in hamster cells infected with SA-7 to screen a large number of industrial chemicals for carcinogenic activity. Hamster cells were exposed to chemicals 18 hr prior to, or 5 hr after infection with SA-7. Salts of arsenic, beryllium, cadmium, chromium, cobalt, iron, lead, manganese, nickel, and platinum significantly increased the transformation frequency and the number of foci per infected culture.
Among the 30 large production scale chemicals tested, only propylene oxide, vinyl acetate, and 2-chloro-1,2-butadiene enhanced transformation. Some inorganic metals, commercial acids, and organic solvents were negative for enhancement of transformation.

A large series of both activation-dependent and activation-independent chemical carcinogens consistently amplified SV40 DNA sequences in Chinese hamster embryo cells transformed with SV40 (Lavi, 1981; 1982; Lavi and Etkin, 1981). Amplification was detected with a highly sensitive in situ hybridization procedure specific for cells synthesizing SV40 DNA. Not all of the viral sequences were present in the integrated SV40 inserts. Infectious virus and/or complete viral DNA molecules were never recovered from treated cells. Some of the chemical carcinogens which amplified SV40 DNA included BP, MNNG, 7,12-DMBA, phenanthrene, 3-MCA, cyclophosphamide, MNU, EMS, dibenz[a,c]anthracene, AB, and BP-7,8-diol-9,10-epoxide (anti). Noncarcinogenic hydrocarbons were inactive in the system. Inhibitors of PAH metabolism, such as 7,8-benzoflavone, prevented amplification of SV40 DNA, indicating a requirement for metabolic activation. The authors suggested that SV40 amplification by chemicals might be valuable as a short-term assay for carcinogens.

Coughill and Moore (1983) developed a mammalian inductest for suspected carcinogens which relied on the induction of SV40 from SV40-transformed hamster kidney cells. Chemical compounds used in the system were ranked as follows from greatest inducing ability to lowest inducing ability: sterigmatocystin, AB, aflatoxin G, 9,12-DMBA, and BP. No induction occurred with 1,2-benzanthracene.
In general, the levels of induction increased directly with the concentrations of chemicals used. BP and 9,12-DMBA required exogenous enzyme activation in the form of S9 mix. It was not determined whether induction occurred as a direct result of damage to the DNA or by indirect factors such as the action of a repair system or the release of a viral repressor. Zamansky et al. (1976) showed that SV40 induction was enhanced by inhibition of post-replication repair. In this case, caffeine, which interferes with the filling of gaps formed during post-UV DNA repair, stimulated the induction of SV40 after UV-irradiation. MMC, which causes DNA strand breakage, is also a potent inducer of SV40 (Rakusanova et al., 1978). Hence, it appears that either direct or indirect DNA damage can lead to induction of SV40.

A modified in vitro transformation assay used by Traul et al. (1981) has potential for the identification and study of tumor promoters. They treated F344 rat embryo cells, infected with Rauscher MuLV, with subeffective doses of 3-MCA. Transformation occurred only when 3-MCA-initiated cells were treated with tumor promoters such as TPA, sodium phenobarbital, limonene, oleic acid, lauric acid, and saccharin. Cultures treated with 3-MCA alone or with tumor promoters alone showed no evidence of focus formation during 8 to 9 culture passages. Focus-positive cultures also formed attachment independent colonies in agarose. Traul et al. (1981) speculated that the virus was not acting as the initiator in this 3-party system, but rather as a modulator of cell membrane transport or of carcinogen metabolism.
Another short-term in vitro assay for detecting tumor promoters used EBV-nonproducer Raji cells and n-butyrate as the inducer (Ito et al., 1981). After the addition of n-butyrate and the promoting agent, the cells were cultivated for 48 hr and the ratio of cells expressing EA was assessed. The chemicals showing positive promoting activity in this system included TPA and related compounds, Euphorbiaceae plant extracts, and some microbial products such as teleocidin. Other skin tumor promoters such as anthralin, phenol, tween-60 and 80, and carcinogenic chemicals such as BP, showed no EA-inducing activity even at maximum doses.

Bockstahler et al. (1982) developed two possible in vitro mammalian host cell-virus systems with potential for identifying chemicals with photosensitizing potential. Infectious SV40 was induced from SV40-transformed hamster cells by treatment with proflavine plus visible light or 8-methoxypsoralen plus near UV-irradiation. These same photosensitizing treatments inactivated the capacity of monkey cells to support the growth of HSV. It was suggested that either SV40 induction from hamster cells or inactivation of monkey host cell capacity for HSV replication might serve as useful screening systems for testing the photosensitizing potential of chemicals.

In summary, current assays for carcinogens which use viruses involve transformation by SA-7 and amplification or induction of SV40 DNA in infected cells. Assays for promoting activity involve EA induction by EBV-non-producer cells or transformation of MuLV-infected cells, initiated with 3-MCA. Current proposed assays for
chemicals with photosensitizing potential involve induction of SV40 or cellular inactivation of HSV production.

Analysis of DNA Repair Processes Using Viruses and Chemical Treatment

Chemicals are valuable tools in the study of DNA repair processes when used in combination with viruses or viral nucleic acid. When monkey kidney cells were pretreated with UV-irradiation or MMC and transfected 24 hr later with intact or UV-irradiated DNA from a temperature sensitive mutant of SV40, the survival of SV40 progeny increased when UV-irradiated viral DNA was used for transfection (Gentil et al., 1982). This type of enhanced reactivation appears to be a general phenomenon and has been reported for different viruses, different cell lines, and for different DNA-damaging treatments. Because native SV40 DNA was used in their study, Gentil et al. (1982) hypothesized that enhanced reactivation could be the consequence of a new recovery pathway allowing for more efficient replication of damaged viral DNA and not the result of alterations in the infection process. Only enhanced reactivation was observed and not enhanced mutagenesis.

Sarasin et al. (1982) analyzed the molecular mechanism of mutagenesis in carcinogen-treated mammalian (monkey kidney) cells using a UV-irradiated SV40 mutant as a biological probe to detect inducible DNA repair and mutagenesis. Pretreatment of the cells with UV-light, 2-AAF, or MMC increased the mutagenesis of UV-irradiated SV40, which was measured as a reversion frequency from temperature-sensitive to wild type phenotype. Increased mutagenesis was not observed with undamaged virus. The authors believed they were looking at targeted
mutagenesis involving single base pair substitution in some cases. Carcinogen treatment of monkey cells appeared to activate some form of error-prone replication which was better able to replicate UV-damaged templates, but which resulted in a higher level of mutagenesis.

When a human kidney carcinoma cell strain was treated with MNNG, N-ethyl-N'-nitro-N-nitrosoguanidine, or MNU, it was less able to support the replication of MNNG-treated adenovirus 5 than untreated virus (Day and Ziolkowski, 1981). It appeared that MNNG pretreatment of the cells was blocking their ability to remove $\text{O}^6$-methyl guanine ($\text{O}^6\text{MG}$) from the MNNG-damaged viral DNA. Two kinds of mechanisms were envisioned which could cause such inhibition: 1) cellular DNA damage might compete for repair with viral DNA damage; and/or 2) direct viral recovery (e.g. by removal of free -SH groups) or indirect viral recovery (e.g. by activation or induction of a protease) might be inactivated.

Su et al. (1981) observed the coordinate expression of mutator and repair activities after exposing human or rat cells to nontoxic concentrations of two different 2-nitronaphthofuran derivatives, many of which are carcinogens. They used unirradiated parvovirus H-1 as a probe for enhanced mutagenesis and UV-irradiated parvovirus H-1 as a probe to demonstrate enhanced reactivation of the virus. This was the first evidence that chemicals could enhance the mutagenesis of undamaged DNA by activating the expression of mutator functions in mammalian cells.
Caffeine, which is an inhibitor of DNA repair, was used to study the potentiating effect of chemical carcinogens on Friend viral leukemogenesis in mice (Raikow et al., 1981). The ability of BP and MMS to increase the incidence of leukemia in mice infected with low levels of Friend MuLV was further enhanced by caffeine. In the absence of carcinogen treatment, caffeine showed no carcinogenic effect either when administered alone or in combination with Friend MuLV.

In summary, chemicals used in combination with damaged SV40 DNA demonstrate enhanced reactivation and activation of error-prone replication pathways. Treatment of cells with the alkylating agent, MNNG, apparently blocks their ability to remove $O^6$MG from MNNG-treated adenovirus 5 DNA. Viral nucleic acid has also been used to detect the coordinate activation of mutator and repair capacities induced by carcinogenic chemicals in mammalian cells. Inhibition of DNA repair may also potentiate chemically induced viral transformation \textit{in vivo}.

Chemical Effects on Cellular Oncogenes

Correlated to the study of viral-chemical interactions is the discovery that cellular oncogenes may be activated or regulated by chemical carcinogens or tumor promoters. Cellular oncogenes may be influenced by chemically mediated processes in a manner analogous to the transforming RNA tumor viruses, most of which originated from the transduction of cellular proto-oncogenes by replication independent retroviruses (Land et al., 1983).
Sukumar et al. (1984) demonstrated an activated ras oncogene in neoplastic cells derived from five independent guinea pig cultures after exposure in vitro or in vivo to four different chemical carcinogens including 3-MCA, BP, MNNG, and DEN. They specifically detected v-bas, which is the onc gene of BALB MSV and a member of the ras gene family of oncogenes. Activation of the ras oncogene was thought to be a necessary step in the acquisition of neoplastic properties by these carcinogen-treated guinea pig cells. This was demonstrated by the fact that early nontumorigenic passages of each carcinogen-treated cell line showed no detectable anchorage-independent growth, and their DNA's did not induce morphologic transformation of NIH/3T3 cells. However, DNA isolated from later cell passages demonstrated transforming activity in NIH/3T3 cells.

The induction of mammary carcinomas by nitroso-methylurea (NMU) involved the specific activation of the H-ras-1 (Harvey MSV) locus by a single point mutation (Sukumar et al., 1983). NMU is a potent alkylating agent which preferentially methylates at N7 and O6 of deoxyguanosine, resulting in deoxyguanosine residues which often pair with thymidine instead of deoxycytidine. This generates G to A transitions. This was precisely the mutation which appeared responsible for malignant activation of the H-ras oncogene by NMU. The twelfth codon of the mutated H-ras gene was GAA instead of GGA of the normal allele, encoding glutamic acid in place of glycine. Oncogene activation was apparently a direct consequence of the interaction of NMU with the DNA.
Sequential treatment of mouse skin with the initiator, DMBA, and the promoter, TPA, induced epidermal tumors. When DNA from three of such carcinomas was used to transfect NIH/3T3 cells, morphologically transformed foci resulted (Balmain and Pragnell, 1983). An additional copy of the activated cellular homolog of the H-ras gene was detected in the transformed foci. The activated H-ras copy was demonstrated to have come from exogenous DNA from the tumor and not by duplication of the endogenous H-ras gene of the NIH/3T3 cells.

Parada and Weinberg (1983) analyzed the genomes of NIH/3T3 fibroblasts after transfection with the DNA from 3-MCA-transformed fibroblasts from mice. Increased copies of the cellular homolog of the Kirsten sarcoma virus ras oncogene were found. The Ki-ras oncogenes may have become activated by the direct action of 3-MCA on the Ki-ras proto-oncogene DNA, or the introduction of 3-MCA into the mouse fibroblasts might have triggered a complex series of events including activation of the Ki-ras oncogene. Eva and Aaronson (1983) similarly observed amplification of the cellular homolog of the ras oncogene of Kirsten MSV in NIH/3T3 cells after transfection with DNA from 3-MCA initiated mouse fibrosarcomas.

Human myeloblastic leukemia (ML-1) cells in a proliferating state normally express c-myb (cellular homolog of the oncogene of avian myeloblastosis virus). When TPA was used to induce differentiation of ML-1 cells, a rapid decline in c-myb expression occurred (Craig and Bloch, 1984). The decrease in c-myb expression was coincident with the time that monocyte and macrophage-like cells were beginning to emerge.
To summarize the effects of chemical carcinogens on cellular oncogenes, transfection studies have demonstrated the activation of the ras oncogenes by chemicals both in vitro and in vivo. Conversely, the tumor promoter, TPA apparently inhibits the expression of c-myb which is normally activated in some human leukemia cells.

Conclusion

It is difficult to determine the precise molecular mechanisms involved in the large majority of chemical-viral interactions discussed. The diverse classes of compounds which have been utilized in combination with viruses probably also allow for an equally diverse spectrum of consequences. If chemicals cause damage to DNA, permanent alterations in cellular or viral control mechanisms may result or host repair processes may be activated. When agents incorporate into host cellular DNA, which contains integrated viral genomes, DNA expression and differentiated functions may be altered. Structural and regulatory genes may also be rearranged. Chemicals could cause a multitude of transcriptional alterations of viral genes or possibly modify target sites for viral gene products.
MATERIALS AND METHODS

Culture of Feline Leukemia Virus - Kawakami-Theilen Strain (KT-FeLV)

KT-FeLV infected FL-74 cells were grown in suspension culture in 0.5 gallon disposable glass roller bottles in complete medium (CM) consisting of RPMI 1640 (GIBCO Laboratories) containing 0.2% sodium bicarbonate plus 15% newborn calf serum (Sterile Systems, Inc.) and supplemented with 1% L-glutamine, 2,000 units penicillin/ml, and 0.1% gentamicin sulfate.

Cells were seeded at 7 day intervals at 1.2 to 1.4 x 10^6 cells/ml in 200 ml of CM per roller bottle and incubated at 37°C on a roller apparatus. On day 5 the cells were fed 200 additional ml of CM per roller bottle and harvested on day 7.

A different culture regimen was also used. FL-74 cells were seeded at 2.5 x 10^6 cells/ml in 7 liters of CM. The cells were suspended with slow stirring in a 15 liter Bellco spinner flask at 37°C. The culture medium containing virus was harvested after 12 hrs of growth.

The cells were collected by centrifugation for 15 min. at 1500 RPM in a GSA rotor of a Sorvall centrifuge at 25°C. Virus was purified from the culture supernatant.
Purification of KT-FeLV

Purification procedures were based on methods of Brian et al., 1975; Thomason et al., 1976; and Thomason et al., 1978 with modifications.

1) Approximately 10 to 15 liters of clarified culture medium were concentrated at 4°C by membrane filtration with a Millipore-Pellicon membrane filter unit (Millipore Corp.) having an exclusion limit of 100,000 molecular weight. Final concentration volume was approximately 800 to 1000 ml.

2) The concentrated culture medium was centrifuged at 25,000 RPM for 1 hr at 4°C in a Beckman SW 27 rotor to concentrate the virus and exclude it from other high molecular weight compounds in the medium (e.g. serum proteins).

3) The viral pellets were resuspended in 1X TNE buffer, pH 7.4 (0.01M Tris, 0.1M NaCl, and 0.001M EDTA) at 4°C. (From the original 15 liters, 12 pellets were obtained and resuspended in 5 ml of 1X TNE per pellet.)

4) The resuspended virus was layered onto 20-50% linear sucrose density gradients prepared in 1X TNE, pH 7.4 and centrifuged at 25,000 RPM for 1 hr at 4°C in the SW 27 rotor. (Five ml virus suspension was layered onto each of twelve 30 ml gradients.)

5) The virus sedimented approximately half way down in the gradients and was harvested manually from above with a cannula and syringe.

6) The combined viral fractions (approximately 150 ml) in sucrose, were dialyzed at 4°C against 1X TNE, pH 7.4. (Two changes
of 6 liters each at 1 day intervals reduced the sucrose concentration to less than 1%.

7) The dialyzed virus was finally concentrated by centrifugation at 25,000 RPM at 4°C in the SW 27 rotor for 1 hr.

8) The virus pellets were suspended in a total of 15 ml of 1X TNE, pH 7.4 and stored frozen at -85°C prior to extraction of RNA.

**Extraction and Purification of RNA from KT-FeLV**

These methods were adapted and modified from those used by Brian et al., 1975; Sherr et al., 1980; and Thomason et al., 1976.

All glassware and buffers were nuclease-free after treatment with diethyl pyrocarbonate (Sigma). All steps were carried out at room temperature unless otherwise indicated.

1) KT-FeLV in 1X TNE, pH 7.4 was made 1.0% with SDS by adding one-tenth volume of 10% SDS in TNE, pH 7.4.

2) An equal volume of proteinase K (Boehringer Mannheim, self-digested at 60°C for 20 min.) at 1000 µg/ml in 1X TNE, pH 7.4 was added (final proteinase K concentration was 500 µg/ml) and the mixture was incubated for 30 min. at 37°C.

3) Two volumes of freshly prepared 1X TNE, pH 7.4 - saturated-phenol were added and the mixture was stirred slowly at room temperature for 30 min. to 1 hr.

4) The mixture was centrifuged at 700 X g in a Beckman model TJ-6 centrifuge for 10 min. at room temperature to separate the phases. The aqueous layer was removed with nuclease-free Pasteur pipets.
5) The aqueous phase was subjected twice more to steps 3 and 4. (Three phenol extractions in total.)

6) The final aqueous phase was made 0.2M with Na Acetate by addition of one-tenth volume of 2M stock Na Acetate (nuclease-free), pH 5.0.

7) Two volumes of cold absolute ethanol (-20°C) were added and the RNA was precipitated during storage at -20°C for at least 24 hrs.

8) The precipitated RNA was collected by centrifugation at 6,000 RPM for 2 hrs in the SS 34 rotor of a Sorvall centrifuge at 0°C.

9) The RNA pellets were carefully decanted, drained and resuspended in 200–300 μl of 1X TNE, pH 7.4.

10) The RNA was layered onto 15-30% linear sucrose (nuclease-free) gradients prepared in 1X TNE, pH 7.4 and centrifuged at 38,000 RPM for 3.75 hrs in the Beckman SW41 rotor at 25°C.

11) Gradients were fractionated and scanned with an ISCO density gradient fractionator and UV monitor.

12) The peak fractions representing the viral 35S RNA and 70S RNA were collected separately and the RNA was precipitated by addition of one-tenth volume of 2.0M Na Acetate and two volumes of absolute ethanol, and storage at -20°C.

13) The precipitated 70S viral RNA was collected as in step 8 and recentrifuged in sucrose gradients (step 10). The 70S viral RNA preparation was isolated after centrifugation and resuspended in 1X TNE, pH 8.3.

14) 70S RNA from step 13 was also resuspended in 0.01N Tris, 0.1N NaCl, 0.0001M EDTA, 0.2% SDS, pH 7.4, and heat denatured in boiling
water for 45 sec. to dissociate it into 35S subunit molecules. The
denatured 70S RNA was subsequently chilled on ice and analyzed in
15-30% sucrose density gradients as described in step 10.

**Preparation of Radiolabelled Complementary DNA (C-DNA) to KT-FeLV RNA**

These methods were adapted from those of Collett and Faras, 1975;
Retzel et al., 1980; Sherr et al., 1979; and Taylor et al., 1976.

The following components were added in order to a final 1 ml
reaction mixture:

Fifty mM Tris-HCl, pH 8.1; 8 mM MgCl₂; 1 mM of a combined mixture
of dATP, dTTP, and dGTP; 20 mM 2-mercaptoethanol; 50 ug of FeLV RNA
(35S or 70S); 2.5 mg calf thymus primers; 80 ug/ml bentonite; 140 mM
KCl; 250 uCi ³²P-dCTP¹; and 750 units of AMV reverse transcriptase.²

Incubation was for 3 hrs at 37°C in capped plastic siliconized,
nuclease-free microfuge tubes.

The oligonucleotides of the calf thymus DNA primer mixture were
generated by digesting 5 mg of calf thymus DNA (Sigma) with 70 µg
DNase I (Sigma) per ml in a reaction mixture containing 10 mM Tris-
HCl, pH 7.4 and 10 mM MgCl₂ at 37°C for 2 hrs. Afterward the DNase I
was inactivated by heating at 121°C for 10 min. The initial DNA
concentration was used as the measure of the primer concentration.
(Active primers were approximately 8-15 nucleotides in length.)

The procedure used to terminate the C-DNA reaction was based on
that of Retzel et al., 1980.

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¹ Amersham (2,000-3,000 Ci/mM specific activity).
² Bethesda Research Laboratories, Inc.
1) Following the 3 hr incubation, an equal volume of 100 mM EDTA, 1% SDS, and 1 mg proteinase K/ml prepared in 50 mM Tris-HCl, pH 8.1 was added and the mixture was incubated for 30 min. at 37°C.

2) This mixture was diluted to 5 ml with 50 mM Tris-HCl, pH 8.1 containing 0.1M NaCl.

3) The 5 ml mixture was extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). The phenol was saturated with 50 mM Tris-HCl, pH 8.1. Extraction was for 30 min. at room temperature with gentle stirring. The mixture was centrifuged at 850 X g in a Beckman Model TJ-6 centrifuge for 15 min. at 25°C and the upper aqueous phase was collected.

4) The aqueous phase was treated for 10 min. at 65°C with 0.25N NaOH to hydrolyze the template RNA. The mixture was then neutralized with HCl.

5) The C-DNA was precipitated by adding 2 volumes of cold absolute ethanol (-20°C) and one-tenth volume of 2M Na acetate, pH 5.0 to the aqueous phase.

6) The single-stranded C-DNA was collected by centrifugation at 6,000 RPM for 2 hrs in the SS34 rotor of a Sorvall centrifuge at 0°C and separated from calf thymus primers and unincorporated nucleoside triphosphates by gel filtration on Sephadex G-50 using 50 mM Tris-HCl, pH 8.1, as the elution buffer.

7) The pure C-DNA was precipitated again at -20°C with ethanol and 0.2M Na acetate and was concentrated by centrifugation at 6,000 RPM for 2 hrs in a Sorvall SS34 rotor at 0°C, resuspended in 200-500 µl of 50 mM Tris-HCl, pH 8.1, and stored at -85°C.
Analysis of KT-FeLV RNA and C-DNA Products in Agarose Gels

This method, based on that of McMaster and Carmichael (1977), employed denaturation of nucleic acids and their reaction with glyoxal, followed by electrophoresis in a horizontal slab gel system. Glyoxalation introduced an additional ring onto guanosine residues which sterically hindered the formation of G-C base pairs and renaturation of native structure, allowing for a more accurate determination of molecular weight.

1) DNA samples were incubated in capped nuclease-treated and siliconized plastic microfuge tubes for 1 hr at 50°C in 1.0M glyoxal (Eastman Kodak Company), 50% (vol/vol) dimethyl sulfoxide (DMSO), and 10 mM NaH$_2$PO$_4$/Na$_2$HPO$_4$, pH 7.0. Control samples were treated identically but in the absence of glyoxal and DMSO. Oxidation products were removed from glyoxal immediately prior to use by passing the glyoxal solution 4 times over a column of mixed bed ion-exchange resin AG-501-X8 (BioRad).

2) After incubation, the samples were cooled to room temperature. Sucrose was added to 5% just before electrophoresis. The samples were electrophoresed in 1.2% agarose horizontal slab gels prepared in 10 mM NaH$_2$PO$_4$/Na$_2$HPO$_4$, pH 7.0, which was also the running buffer. Electrophoresis was at a constant current of 45 mAmps for 2 hrs at room temperature. The marker dye was 0.25% bromophenol blue in 50% glycerol. DNA molecular weight markers were the Eco R I and Hind III digests of Lambda DNA (Boehringer Mannheim).

3) Gels were stained for 20 min. with 15 μg/ml acridine orange (Sigma) in 10 mM Na phosphate buffer, pH 7.0, and destained
overnight. Gels were viewed for fluorescence on a UV Fotodyne Trans-
iluminator (Fotodyne Incorporated) at 254 nm wavelength and photo-
graphed.

Culture of Human Foreskin Fibroblast Cells (Detroit 550), American
Type Culture Collection (CCL 109)

Monolayer cultures were grown in Corning 490 cm$^2$ tissue culture
roller flasks on a roller apparatus at 37°C in Minimum Essential
Medium - Earle's Salts (MEM-E; GIBCO Laboratories) containing
0.22% sodium bicarbonate plus
10% fetal calf serum (Sterile Systems, Inc.) and supplemented
with
1% non-essential amino acids,
1% sodium pyruvate,
1% L-glutamine, and
0.1% gentamicin sulfate
(100 ml per flask).

Approximately 6 x 10$^6$ cells were seeded per roller flask and
grown to confluency. The cells from each confluent flask were
passaged into 2 to 3 roller flasks until adequate numbers were grown
for co-carcinogenesis transformation studies.

Co-Carcinogenesis Transformation of Detroit 550 (D-550) Cells

Forty (490 cm$^2$ tissue culture) roller flasks containing 10 x 10$^6$
cells/flask and between passages 20 to 30 were used for each
transformation experiment to provide sufficient cellular DNA for
hybridization experiments. Following are the chemicals which were
administered, their concentrations, and time of administration
relative to infection with ST-FeSV at $6.0 \times 10^3$ focus-forming units/flask. ST-FeSV was titrated and donated by Mrs. Ann Elliot (The Ohio State University, Columbus, OH). Chemicals were obtained from Aldrich Chemical Co.).

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Concentration</th>
<th>Relative to ST-FeSV Infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrazine</td>
<td>60 ppm</td>
<td>-2 and +2 hours</td>
</tr>
<tr>
<td>Mono-methyl Hydrazine</td>
<td>100 ppm</td>
<td>-2 and +2 hours</td>
</tr>
<tr>
<td>Symmetrical Dimethyl Hydrazine</td>
<td>100 µg/ml</td>
<td>-2 and +2 hours</td>
</tr>
<tr>
<td>Unsymmetrical Dimethyl Hydrazine</td>
<td>100 µg/ml</td>
<td>-2 and +2 hours</td>
</tr>
<tr>
<td>Benzo[a]pyrene</td>
<td>10 µg/ml</td>
<td>-2 and +2 hours</td>
</tr>
<tr>
<td>Pyrene</td>
<td>10 µg/ml</td>
<td>-2 and +2 hours</td>
</tr>
<tr>
<td>Phenyl-α-Naphthylamine</td>
<td>10 µg/ml</td>
<td>+2 hours</td>
</tr>
</tbody>
</table>

Detroit 550 cells - untreated with chemicals and uninfected, and D-550 cells infected with ST-FeSV only were controls.

The procedures for co-carcinogenesis transformation of D-550 cells were as follows:

I. Procedure for administration of chemical 2 hrs prior to ST-FeSV infection:
   1) Medium was aspirated from roller flasks.
   2) Twenty ml of chemical was added at the desired concentration in complete MEM-E medium containing 10% fetal bovine serum (FBS) to each roller flask and incubated at 37°C for 1.5 hrs.
3) The chemical was aspirated from the flasks which were washed with complete MEM-E.

4) DEAE-Dextran (Sigma) was added at 40 μg/ml prepared in incomplete MEM-E medium (no FBS) using 5 ml per culture flask. (DEAE-Dextran enhances virus absorption and penetration into the cells by altering the cell surface charge characteristics.) Incubation was at 37°C for 20 min.

5) DEAE-Dextran was aspirated from the flasks.

6) D-550 cells were infected with ST-FeSV at approximately 1.22 x 10³ focus-forming units/ml (FFU/ml) in 5 ml MEM-E medium containing 5% FBS per culture flask. Incubation was at 37°C for 2 hrs.

7) Virus was aspirated from the flasks.

8) Fifty ml of complete MEM-E medium (10% FBS) was added to each culture flask. Cells were grown an additional 6 days at 37°C.

9) Cells were harvested by trypsinization, pelleted by low speed centrifugation (200 x g for 10 min. in a Beckman Model TJ-6 centrifuge), and stored frozen at -85°C.

II. Procedure for administration of chemical 2 hrs after ST-FeSV infection.

1) Medium was aspirated from the roller flasks.

2) DEAE-Dextran at 40 μg/ml was prepared in incomplete MEM-E medium (no FBS) using 5 ml per culture flask. Incubation was at 37°C for 20 min.

3) DEAE-Dextran was aspirated from the flasks.
4) Cells were infected with ST-FeSV at $1.22 \times 10^3$ FFU/ml in MEM-E medium containing 5% FBS using 5 ml per culture flask. Incubation was at 37°C for 2 hrs.

5) Virus was aspirated from the flasks.

6) Twenty ml of chemical was added at the desired concentration in complete MEM-E medium containing 10% FBS to each roller flask and incubated at 37°C for 1.5 hrs.

7) The chemical was aspirated from the flasks which were then washed with 5 ml complete MEM-E.

8) Fifty ml of complete MEM-E medium (10% FBS) were added to each culture flask. The cells were grown 6 additional days at 37°C.

9) Cells were harvested by trypsinization, pelleted by low speed centrifugation (200 X g for 10 min. in a Beckman Model TJ-6 centrifuge) and stored frozen at -85°C.

Purification of Nuclear and Cytoplasmic DNA from D-550 Cells

I. Separation of Nuclear and Cytoplasmic Fractions from D-550 Cells

(Methods were modified from those of Ghosh et al., 1980).

The cell pellet (approximately 4-5 ml in volume) was suspended in 30 ml of hypotonic buffer consisting of 0.01M Tris-HCl, pH 7.4, 0.01M NaCl, and 0.003M MgCl$_2$. The cells were allowed to swell for 20 min. Ten ml of a mixture of 6.7% Tween-40 (v/v) (Sigma) and 3.3% Na deoxycholate (w/v) (Sigma) were added and the cells were lysed with 10-20 strokes in a tight fitting Dounce homogenizer. This resulted in complete lysis of cells without apparent lysis of nuclei.

The cell lysate was centrifuged for 10 min. at 4,000 RPM in a Sorvall SS34 rotor at 4°C. The cytoplasmic supernatant was decanted
and saved. The nuclei were resuspended in hypotonic buffer and recentrifuged (10 min. at 4,000 RPM). The second cytoplasmic supernatant fraction was combined with the first.

The nuclear pellet was then taken up in 10 ml of hypotonic buffer and 1.5 ml of 6.7% Tween-40 (v/v) and 3.3% Na deoxycholate (w/v) was added. This suspension was vortexed for 2-3 seconds and the nuclei were sedimented a third time (10 min. at 4,000 RPM in the Sorvall SS34 rotor at 4°C). The nuclei were resuspended in hypotonic buffer and resedimentated a fourth time. This freed the nuclei of essentially all traces of cytoplasmic material. The four cytoplasmic fractions were combined and frozen at -85°C.

II. Extraction and Purification of High Molecular Weight DNA from Nuclei of D-550 Cells. (Methods were modified from those of Britten et al., 1974 and Fujinaga et al., 1973).

All glassware used throughout the extraction was siliconized and treated with diethyl pyrocarbonate to remove nucleases.

1) The nuclear pellet (from 40-490 cm² roller bottles) was resuspended in 9 ml of 1X TNE buffer, pH 8.0.

2) One-tenth volume (1 ml) of 5.0% SDS prepared in TNE, pH 8.0 was added to the nuclear suspension to disrupt the nuclear membranes.

3) An equal volume (10 ml) of proteinase K (self-digested at 60°C for 20 min.) at 1000 µg/ml was added (500 µg/ml final concentration) and the mixture was incubated at 37°C for 2 hrs.

4) The digested mixture was extracted three separate times with an equal volume of TNE, pH 8.0 - saturated-phenol:chloroform:isoamyl
alcohol (25:24:1). Each extraction was stirred slowly at room temperature for 30 min.

5) The phases were separated by centrifugation at 850 X g in a Beckman Model TJ-6 centrifuge for 15 min. at 25°C. The upper aqueous phase was collected from each extraction with siliconized Pasteur pipets.

6) DNA was precipitated from the final aqueous extract by addition of 2 volumes of cold, absolute ethanol (-20°C) and one-tenth volume of 2M Na acetate, pH 5.0 and storage for approximately 48 hrs at -20°C.

7) The precipitated DNA was spooled out on a siliconized glass rod and resuspended in 4 ml of TNE buffer, pH 8.0. The DNA was sonicated on ice for 1 min. using 3 separate 20 sec. bursts from a Branson Sonifier Cell Disrupter 200 (Branson Sonic Power Co.). DNA was sonicated to enhance solubility and to reduce the size length to approximately 5,000-2,000 base pairs for increased hybridization efficiency.

8) The sonicated DNA was digested with an equal volume of RNase A (boiled 1 min.) (Sigma) at 100 μg/ml (50 μg/ml final concentration). Incubation was at 37°C for 2 hrs.

9) The RNase-treated DNA was then digested with nuclease-free pronase (self-digested at 37°C for 1 hr) (Calbiochem-Behring Corp.) at a final concentration of 100 μg/ml for 2 additional hrs at 37°C.

10) RNA was hydrolyzed by heating at 65°C for 10 min. with 0.25M NaOH.
11) After neutralization with HCl, the DNA was extracted with 2 volumes of TNE, pH 8.0 - saturated-phenol:chloroform:isoamyl alcohol (25:24:1).

12) DNA was precipitated from the aqueous phase by addition of 2 volumes of cold absolute ethanol (-20°C) and one-tenth volume of 2M Na acetate, pH 5.0, and storage at -20°C for 48 hrs.

13) The nuclear DNA was collected by centrifugation for 2 hrs at 6,000 RPM using the Sorvall HB-4 rotor in a Sorvall centrifuge at 0°C and resuspended in 200 µl of 1X TE buffer, pH 8.0 (0.01N Tris-HCl, 0.001M EDTA).

14) The DNA was quantitated by measuring its optical density at 260 nm (50 µg/ml of double stranded DNA is approximately equal to 1 O.D. at 260 nm) and its purity was determined by the 260/280 nm ratio which should be 1.8 for pure DNA. In addition DNA was quantitated by a second method which involved dilution and mixing with an equal volume of ethidium bromide (EtBr) at 2 µg/ml. The fluorescence was compared to a dilution series of calf thymus DNA standards, (Maniatis et al., 1982, p. 468-469).

III. Extraction and Purification of Cytoplasmic DNA from D-550 Cells.

The methods used for purification of cytoplasmic DNA were similar to those used for nuclear DNA with the following modifications:

1) To reduce volume and quantities of proteinase K required for digestion, the cytoplasmic fractions were initially extracted at room temperature with 2 volumes of TNE-saturated-phenol:chloroform:isoamyl alcohol (25:24:1). One-tenth volume of 5% SDS in TNE, pH 8.0 was also added prior to extraction. The upper aqueous phase was separated
by centrifugation at 850 X g for 15 min. at 25°C and the DNA was precipitated at -20°C after addition of 2 volumes of absolute ethanol (-20°C) and one-tenth volume of 2M Na acetate, pH 5.0.

2) The precipitated DNA was collected by centrifugation at 6,000 RPM for 2 hrs at 0°C in the SS34 rotor of a Sorvall centrifuge. This DNA was resuspended in 20 ml of 1x TNE, pH 8.0 and treated with an equal volume of proteinase K at 1000 µg/ml (500 µg/ml final concentration) for 2 hrs at 37°C. Cytoplasmic DNA was not sonicated as it was already of sufficiently low molecular weight for hybridization. After two extractions with TNE-saturated-phenol:chloroform:isoamyl alcohol (25:24:1) and precipitation at -20°C, treatment of cytoplasmic fractions was identical to that of nuclear fractions including treatment with RNase A, pronase, and RNA hydrolysis. Cytoplasmic DNA was digested with RNase A at a final concentration of 100 µg/ml (twice that used for nuclear DNA) due to larger amounts of contaminating RNA in the cytoplasmic fractions. Quantitation was the same as that used for nuclear DNA.

Propagation of Plasmid DNA

E. coli strain D5410 (bearing plasmid pBR322) was provided by Dr. John Reeve (The Ohio State University, Columbus, OH).

E. coli strains D5410 and K802 (bearing cloned v-fes in pBR322) were propagated in Luria-Bertani (LB) medium consisting of 10 gm Bactotryptone, 5 gm Bacto yeast extract (DIFCO Laboratories), and 5 gm NaCl per liter. Ten mM MgCl2 and 10 µg/ml tetracycline were included in the selective medium used to grow the Sₐ or Sₐ subclones
of v-fes. Fifty µg/ml ampicillin was added to the LB medium used to propagate strain D5410.

The E. coli strains were grown in shaker culture in 400 ml volumes in the dark at 37°C for 24 hrs or until the cultures reached log phase (approximately 4 x 10^8 cells/ml). Chloramphenicol at 200 µg/ml was added after 24 hrs to amplify plasmid DNA replication and to inhibit bacterial chromosome DNA synthesis (Clewell, 1972) and cultures were grown for 24 more hours.

The various strains of E. coli were stored in LB medium plus 20% glycerol in liquid nitrogen.

**Purification of Plasmid DNA**

These methods were adapted from those used by Dr. John Reeve (The Ohio State University, Columbus, OH - personal communication) and from those presented by Davis et al., 1980.

1) Approximately 1200 ml of culture fluid containing E. coli and plasmid DNA were clarified by centrifugation at 5,000 RPM for 20 min. in the GSA rotor of a Sorvall centrifuge at 4°C.

2) The cell pellets were resuspended in a total of 40 ml of 25% sucrose in 0.05M Tris-HCl, pH 8.0.

3) Twenty ml of cell suspension was placed in two separate plastic centrifuge tubes and centrifuged at 10,000 RPM for 10 min. in the SS34 rotor of a Sorvall centrifuge at 4°C.

4) Each of the two cell pellets was resuspended in a total of 4 ml 25% sucrose in 0.05M Tris-HCl, pH 8.0.
5) 0.8 ml of lysozyme (Sigma) at 20 mg/ml (prepared fresh) in 0.25M Tris-HCl, pH 8.0 was added to each tube and the mixture was swirled on ice for 5 min.

6) 1.6 ml of EDTA (Sigma) in 0.25M Tris-HCl, pH 8.0 was then added to each tube and the mixture was swirled on ice for an additional 5 min.

7) Finally, 6.4 ml of lytic mix consisting of 2% Triton X-100 (Sigma), 50 mM Tris-HCl, pH 8.0, and 50 mM EDTA was added to each tube and the lysate was allowed to clear for 20 min. on ice. If the mixture did not clear well, it was heated at 37°C for approximately 1 min.

8) The cleared lysates were centrifuged for 1.5 hr at 15,000 RPM at 4°C in the SS34 rotor of a Sorvall centrifuge to pellet chromosomal DNA from E. coli. The clear supernatant contained plasmid DNA and was recovered with a Pasteur pipet.

9) For every ml of the supernatant, 1 gm of cesium chloride (CsCl) (Bethesda Research Laboratories) was added and the mixture was swirled gently to dissolve the salt.

10) 0.8 ml of EtBr (Sigma) at 10 mg/ml in distilled H₂O was added for each 10 ml of CsCl solution, and the mixture was kept out of direct light. This procedure resulted in a final density of 1.55 gm/ml (η = 1.380) CsCl and the EtBr concentration was approximately 600 µg/ml.

11) The equilibrium density gradients were centrifuged for at least 48 hrs at 41,000 RPM in the SW 50.1 rotor of a Beckman, Model L8-80 ultracentrifuge at 15°C.
12) The DNA-containing bands were illuminated with long-wave-length UV-radiation. The upper band contained linear, bacterial DNA and nicked, circular plasmid DNA. The lower band contained covalently closed supercoiled plasmid DNA and was collected by puncturing the tube with a 20-gauge syringe needle.

Top band = Relaxed DNA (ρ = 1.55 gm/ml).
Bottom band = Native superhelical DNA (ρ = 1.59 gm/ml).

13) EtBr was removed from the plasmid DNA in CsCl by adding 1 volume unit of isopropanol saturated with aqueous 5M NaCl, 10 mM Tris, and 1 mM Na₂EDTA, pH 8.5. The mixture was shaken vigorously and the phases were separated on ice. The colored EtBr phase was discarded. The extractions were repeated until all visible color was removed and the DNA was then extracted one more time.

14) Two volume units of H₂O and 6 volume units of cold, absolute ethanol (-20°C) were added to the DNA and the mixture was placed at -20°C for one to several days to precipitate the DNA.

15) The DNA was sedimented by centrifugation at 6,000 RPM for 2 hrs in the HB-4 rotor of a Sorvall centrifuge at 0°C.

16) The plasmid DNA was resuspended in 5 ml of 1X TE buffer, pH 8.0 and digested with an equal volume of nuclease-free pronase (Calbiochem-Behring Corp.) at 200 μg/ml (100 μg/ml final concentration) for 2 hrs at 37°C.

17) The digested DNA was extracted with 2 volumes of TE-saturated-phenol:chloroform:isoamyl alcohol (25:24:1) for 30 min. at room temperature. The phases were separated by centrifugation at 850 X g for 15 min. at 25°C and DNA was precipitated from the upper aqueous
phase by addition of 2 volumes of cold, absolute ethanol (−20°C) and one-tenth volume of 2M Na acetate, pH 5.0, and storage at −20°C for at least 48 hrs.

18) The plasmid DNA was centrifuged a second time in equilibrium density gradients as described in steps 9-15 and dissolved in 100-200 μl of 1X TE, pH 8.0 and stored at 4°C.

Restriction Endonuclease Digestion and Purification of v-fes DNA

The restriction endonuclease digestion was based upon procedures presented by T. Maniatis et al., 1982, pp. 104-106. Restriction endonuclease digestion was in a 1 ml reaction mixture.

1) Fifty μg of pBR322 DNA containing the S_L or S_R subclones of v-fes in 1X medium salt restriction endonuclease buffer (500 mM NaCl, 100 mM Tris-HCl, pH 7.5, 100 mM MgSO_4_, and 10 mM dithiothreitol (DTT; Sigma), was digested with 50 units of Pst I restriction enzyme (Miles Laboratories, Inc.) during incubation at 35°C for 1.5 hrs.

2) The reaction was terminated by addition of 10 mM EDTA, pH 7.5.

3) The mixture was extracted once with TE-saturated-phenol: chloroform:isoamyl alcohol (25:24:1) and once with chloroform: isoamyl alcohol (24:1) for 30 min. at room temperature. The aqueous phases were collected by centrifugation at 850 X g for 15 min.

4) DNA was precipitated from the aqueous phases by addition of two volumes of absolute ethanol (−20°C) and one-tenth volume of Na acetate, pH 5.0 and storage at −20°C for one to several days.
5) Precipitated DNA was sedimented by centrifugation at 30,000 RPM for 30 min. in the SW 50.1 rotor of a Beckman ultracentrifuge, Model L8-80, at 0°C and resuspended in 0.5 ml of 1X TE, pH 8.0.

6) 0.5 ml of restricted DNA was mixed with 0.3 ml of EtBr (10 mg/ml in H₂O) and 0.4 ml aliquots were layered onto 15-30% sucrose density gradients and centrifuged for 5.25 hrs at 15° in the SW 50.1 rotor of a Beckman ultracentrifuge, Model L8-80, at 40,000 RPM.

7) The top DNA band containing cleaved v-fes DNA was viewed with long-wavelength UV-radiation and collected with a cannula and syringe or by puncturing the tube with a 20-gauge syringe needle.

8) EtBr was removed from the sucrose fraction containing v-fes by addition of CsCl (1 gm/ml) and extraction with equal volumes of isopropanol (saturated with aqueous 5M NaCl, 10 mM Tris, and 1 mM Na₂EDTA, pH 8.5). Extractions were repeated until all visible color was removed. Two volume units of H₂O and 6 volume units of absolute ethanol were added and the DNA was allowed to precipitate at -20°C for 1 to several days.

9) v-fes DNA was collected by centrifugation at 30,000 RPM for 30 min. at 0°C in the Beckman SW 50.1 rotor and was resuspended in 25 μl of 1X TE, pH 8.0. The concentration was determined by visual comparison of EtBr fluorescence with that of known quantities of calf thymus DNA standards at 254 nm. Approximately 1.0 μg of v-fes DNA was recovered from 50 μg of starting plasmid DNA containing the S₅ subclone.

10) The purity of the v-fes DNA was evaluated by electrophoresis in 1% agarose gels prepared and run in 10 mM sodium phosphate buffer,
pH 7.0, for 1.5 hrs at 45 mAmps constant current. Comparisons were made with DNA from unrestricted pBR322 containing the $S_L$ subclone, with pBR322, and with the lower band in sucrose density gradients containing Pst I restricted linear pBR322 DNA. The Hind III digest markers from Lambda DNA (Boehringer Mannheim) were also used for comparison. pBR322 has only one Pst I site into which the Pst I fragments of v-fes were cloned. Hence, Pst I digestion of a pure preparation of pBR322 containing the $S_L$ subclone resulted in two fragments - one being linear pBR322 and the other being the $S_L$ subclone of v-fes DNA.

Radiolabelling of Plasmid and v-fes DNA by Nick-Translation

This procedure was used without modification as reported by Davis et al., 1980, pp. 168-172.

The following reagents making up a total volume of 25 µl were added in order to a nuclease-free, 1.5 ml capacity microcentrifuge tube:

1) 13.1 µl of 1X TE buffer, pH 8.0.

2) 2.5 µl of 10X nick-translation buffer (0.5M Tris-HCl, pH 7.5, 0.1M MgSO$_4$, 10 mM DTT, and 500 µg/ml of Bovine Serum Albumin [BSA - Calbiochem-Behring Corp.]).

3) 2.5 µl of a solution containing 0.2 mM each dATP, dGTP, and dTTP (Calbiochem-Behring Corp.).

4) 1 µl of v-fes or plasmid DNA containing 0.5-1.0 µg of DNA.

5) 0.5 µl of diluted DNase (P.L. Biochemicals). DNase was prepared as follows:
a) DNase at 1 mg/ml was prepared in 50 mM Tris (pH 7.5), 10 mM MgSO₄, 1 mM DTT, and 50% glycerol (stock solution) and stored at -85°C.

b) 0.5 μl of DNase stock was diluted to 100 μl in 1X nick-translation buffer (50 mM Tris, pH 7.5, 10 mM MgSO₄, 1 mM DTT, and 50 μg/ml of BSA) at 0°C.

c) 0.5 μl of 1X diluted stock was diluted to 100 μl in 1X nick-translation buffer at 0°C for use in the nick-translation reaction (total dilution 1/40,000). 2X diluted DNase was stored in small aliquots at -85°C.

6) Five μl of deoxycytidine 5'-α-³²P triphosphate in aqueous solution containing 10 μCi/ul (specific activity was 2,000-3,000 Ci/M, Amersham).

7) 0.4 μl of homogeneous E. coli DNA polymerase I (Bethesda Research Laboratories) containing 1-2 units of enzyme.

The reaction mixture was incubated at 14°C for 3 hrs, and stopped by the addition of 25 μl of 0.02M Na₂EDTA, 2 mg/ml of sonicated salmon sperm DNA (Calbiochem-Behring Corp.) as carrier, and 0.2% SDS.

The mixture was placed on ice and diluted up to 500 μl with 1X TE, pH 8.0. One μl of 0.25% bromophenol blue in 50% glycerol was added as a tracking dye. The reaction was loaded onto a 0.7 x 20 cm Sephadex G-50 (medium) (Pharmacia Fine Chemicals) Econo-column (BioRad) pre-equilibrated with 1X TE, pH 8.0.

Ten to 12 drop effluent fractions were serially collected and the location of the ³²P-labelled DNA was determined by spotting 2 μl samples of each fraction on 3MM filter paper (Whatman Limited) and
counting in BBOT (Packard Instrument Company, Inc.) cocktail in a Beckman liquid scintillation counter. The fractions comprising the first peak of $^{32}$P activity were combined and stored at 4°C.

**Dot-Blot Hybridization of DNA from Chemically Treated D-550's Infected with ST-FeSV to v-fes or pBR322 DNA**

This procedure was based on the methods of Kafatos et al., 1979 and Thomas, 1980.

I. Hybridization of DNA Using Formamide.

1) GeneScreen hybridization transfer membrane (New England Nuclear-DuPont) was cut into approximately 5 x 7.0 inch sheets and prewetted in 10X SSC (1.5M NaCl; 0.15M Na citrate) for 20-30 min.

2) The GeneScreen membrane was dried between two sheets of 3 MM chromatography paper (Whatman) at 37°C.

3) Purified cellular DNA was denatured by heating in a water bath at 90-100°C for 10 min. Doubling dilution series of the denatured DNA were immediately prepared in 10X SSC (1.5M NaCl; 0.15M Na citrate). Ten µl samples of each dilution were spotted onto the GeneScreen membrane with a pipetman (Rainen Instrument Co.). (Approximately 40-45 spots or dilutions were placed onto a membrane of 5 x 7.0 inch dimensions.)

4) The spotted membrane was dried at room temperature and the DNA was fixed by baking it at 80-100°C for 2-4 hrs in a drying oven (Thelco, Model 15, Precision Scientific Co.).

5) The membrane was prehybridized with 50% formamide (deionized)$^3$, 2X SSC$^4$, 0.05M Na$_2$HPO$_4$/NaH$_2$PO$_4$, pH 6.5, 0.02% polyvinyl-pyrrolidone (mol. wgt. 40,000, Sigma), 0.02% bovine serum albumin
(Calbiochem-Behring Corp.), 0.02% Ficoll (mol. wgt. 400,000, Sigma), and denatured salmon sperm DNA\(^5\) (200 \(\mu\)g/ml, Calbiochem-Behring Corp.). Prehybridization was in a heat sealable plastic pouch (Kapak/Scotchpak, Kapak Corp.) with constant agitation for 20 hrs.

6) The prehybridization solution was replaced with 18 ml of 50% formamide (deionized), 2X SSC, 0.05M \(\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4\), pH 6.5, 0.02% polyvinyl-pyrrolidone (mol. wgt. 40,000, Sigma), 0.02% bovine serum albumin, 0.02% Ficoll (mol. wgt. 400,000, Sigma), denatured salmon sperm DNA (200 \(\mu\)g/ml) plus 18 \(\times\) \(10^6\) dpm of \(^{32}\text{P}\)-labelled v-fes or plasmid probe which was denatured at 90-100°C for 10 min. (approximately 1 \(\times\) \(10^6\) dpm was used per 1 ml of hybridization solution). Hybridization was for 48-50 hrs with constant agitation at 42°C.

7) The membrane was washed two separate times with 100 ml of 0.3M sodium chloride, 0.06M Tris-HCl, pH 8.0, and 0.002M EDTA for 10 min. at 25°C with constant agitation.

8) The membrane was then washed 2 separate times with 100 ml of 0.3M sodium chloride, 0.06M Tris-HCl, pH 8.0, 0.002M EDTA, and 0.5% SDS for 1 hr at 60°C with constant agitation.

9) Finally, the filter was washed two separate times with 100 ml of 0.003M Tris base for 1 hr at 25°C with constant agitation.

\(\text{Formamide (Bethesda Research Laboratories, Redistilled, Ultrapure) was deionized by mixing 100 ml with approximately 5 gms of AG 501-X8 (D) mixed bed resin. The mixture was stirred for 30 min. at room temperature, filtered to remove resin, and stored in 10 ml aliquots at -20°C.}\)

\(2\times\text{SSC}: 0.3\text{M sodium chloride}
\quad 0.03\text{M sodium citrate.}\)

\(\text{Salmon sperm DNA (Calbiochem-Behring Corp.) was denatured in water by heating at 90-100°C for 10 min.}\)
10) The membrane was dried at room temperature and used for autoradiography.

II. Hybridization of DNA Using Dextran Sulfate as a Hybridization Enhancer.

1) The membrane was prehybridized with 10 ml of 50% formamide (deionized), 0.2% polyvinyl-pyrrolidone (mol. wgt. 40,000), 0.2% bovine serum albumin, 0.2% Ficoll (mol. wgt. 400,000), 0.05M Tris-HCl, pH 7.5, 1M NaCl, 0.1% sodium pyrophosphate (Sigma), 0.1% SDS, 10% dextran sulfate (mol. wgt. 500,000, Sigma), and denatured salmon sperm DNA (100 µg/ml) at 42°C with constant agitation for 20 hrs.

2) After prehybridization, 2-3 ml of 50% formamide (deionized), 0.2% polyvinyl-pyrrolidone (mol. wgt. 40,000), 0.2% bovine serum albumin, 0.2% Ficoll (mol. wgt. 400,000), 0.05M Tris-HCl, pH 7.5, 0.1% sodium pyrophosphate, 0.1% SDS, and denatured salmon sperm DNA (100 µg/ml) were added plus 12 x 10^6 dpm of ^32P-labelled plasmid or v-fes probe which was freshly denatured at 90-100°C for 10 min. Hybridization was at 42°C for 48 hrs with constant agitation.

3) Washing and drying of the membrane was described in steps 7-10 of the preceding section.

Autoradiography of DNA Blotted on GeneScreen Hybridization Transfer Membranes

These procedures were based on those of Dr. H. G. Kung (Department of Biochemistry, Michigan State University, East Lansing, MI - personal communication).

1) After hybridization and drying, the membranes were placed between Saran wrap.
2) Kodak X-Omat AR film (XAR-5, Eastman Kodak Company) was exposed to the membrane for 24 hrs or longer at -85°C. Dupont Cronex Lightning - Plus 2C intensifying screens were used without blockers.

3) After exposure the film was developed as follows:

5 min. in Kodak liquid X-ray developer.
1 min. in 3% acetic acid stop bath.
10 min. in Kodak rapid fixer.
15 min. in running water.

4) The developed films dried at room temperature and the relative intensities of the DNA spots were used to quantitate genome numbers of v-fes in D-550 DNA.

Quantitation of Genome Numbers of v-fes Present in Human Fibroblast Nuclear DNA

I. A standard series of copy numbers of v-fes were prepared in denatured salmon sperm DNA (Calbiochem-Behring).

Mammalian diploid nuclear DNA has an average molecular weight of $4 \times 10^{12}$ d (Gelb et al., 1971). The $S_L$ subclone of v-fes is about 500 base pairs or $0.33 \times 10^6$ d mol. wgt. The copy standards contained unrestricted v-fes DNA in pBR322 plasmid DNA.

One hundred ug of DNA containing 100 copies of v-fes per average diploid genome was prepared as follows:

1) $S_L = \text{approximately 500 base pairs (bp) or } 0.33 \times 10^6 \text{ daltons (d)}$

(1000 bp = $6.6 \times 10^5$ daltons, for duplex DNA (Davis et al., 1980)

$pBR322 = 4,862 \text{ bp}$

2) $S_L = \frac{500 \text{ bp}}{4,862 \text{ bp}} = 0.1028$ of the total of pBR322 and $S_L$ present in the mixture.
Hence, for every 1 µg of \( S_L \), there was \( 1 / 0.1028 \) or 9.7276 µg of pBR322 DNA.

3) 100 copies of \( S_L \) = 100 \( (0.33 \times 10^6 \text{ d}) \) = 33 \( 10^6 \) d.

4) \[
\frac{100 \text{ copies of } S_L}{1 \text{ diploid mammalian genome}} = \frac{33 \times 10^6 \text{ d}}{4 \times 10^{12} \text{ d}} \times 100 \mu g.
\]

5) 100 copies of \( S_L \) = \( 8.25 \times 10^{-4} \) µg per 100 µg total DNA.

6) pBR322 makes up \( \frac{0.00825}{0.1028} \) or .008025 µg of background DNA.

7) Preparation of 100 µg of DNA containing 100 copies of \( S_L \) (v-fes) per diploid mammalian genome required 0.00885 or \( 8.85 \times 10^{-3} \) µg of unrestricted pBR322 DNA containing \( S_L \) plus 99.99115 µg of denatured salmon sperm DNA.

Copy number standards from 2 copies to 300 copies of v-fes per mammalian diploid genome were prepared. Each copy standard was spotted in 10 µl containing 100 µg of DNA. Comparisons of hybridization to v-fes were made visually as follows:

Doubling dilutions of human fibroblast DNA were spotted onto the membranes in 10 µl quantities. Hybridization to v-fes resulted in a series of relative exposure intensities on the exposed X-ray film. Quantitative comparisons were made when the intensities of the hybridized human nuclear DNA matched that of the copy standards.

For example: If the intensity of 100 µg of the copy standard containing 10 copies of v-fes per genome matched the intensity of 50 µg of human DNA (spotted in 10 µl equal volumes and equal surface areas), then the human DNA was calculated to contain approximately \( \frac{100}{50} = 2 \times 10 \) or 20 copies.
II. Copy standards containing cloned \( S_L \) and salmon sperm DNA were no longer used when it was discovered that \( v-fes \) was not restricted adequately from pBR322 (Dr. Mariano Barbacid, NCI, Bethesda, MD - personal communication). Hybridization of \(^{32}P-v-fes\) with the copy standards was in excess of that specific for \( v-fes \) due to background homology between the pBR322 sequences in the copy standards and in the probe.

Normal, uninfected D-550 fibroblast DNA contains two copies of the cellular homologue of \( v-fes \) per diploid genome (Franchini et al., 1982), (Figure 2). The human \( c-fes \) locus was localized on chromosome 15 (Dalla-Favera, 1982; HeisterKamp, 1982) and sublocalized to the distal end of the long arm of chromosome 15 within bands q 25-26 (Harper et al., 1983). The human \( c-fes \) locus includes both coding and intervening sequences and spans about 3.4 kilobases, with all three introns located in the 5' half of the \( c-fes \) gene. The human \( c-fes \) locus appears similar in complexity to the cat \( c-fes \) locus (Franchini et al., 1982).

DNA from chemically-treated and ST-FeSV-infected D-550 cells was compared with normal D550 DNA to quantitate genome copy numbers of \( v-fes \). Comparisons of DNA blots on autoradiograms were made visually and with a computer assisted - Zeineh soft laser-scanning densitometer (LKB Instruments, Inc.). Quantitation of \( v-fes \) hybridization was done by integrating peak areas resulting from densitometer tracings. DNA blots were also cut out and counted in a Beckman liquid scintillation counter in BBOT cocktail (Packard) after hybridization for determination of genome copy numbers.
Agarose Gel Electrophoresis of Plasmid and Nuclear DNA

Electrophoresis was used to assess the purity of v-fes and plasmid DNA preparations and to isolate restriction fragments of nuclear DNA for use in Southern blotting.

DNA was electrophoresed in 1% agarose gels (Seakem LE Agarose, FMC Corporation, Marine Colloids Division) prepared in 10 mM NaH₂PO₄/Na₂HPO₄ buffer, pH 7.0. Electrophoresis was in a horizontal, flat bed gel apparatus at 45 mVmps constant current for 1 hr and 45 min. in 10 mM Na phosphate, pH 7.0. Gels were stained with acridine orange (15 µg/ml for 20 min.) (Sigma) or EtBr (0.5 µg/ml for 45 min.) (Sigma) prepared in 10 mM Na phosphate buffer, pH 7.0.

Gels stained with acridine orange were destained overnight. EtBr gels were not destained. DNA bands were viewed with a Fotodyne UV transilluminator and photographed using Kodak Kodacolor 35mm print film and a yellow blocking filter.

Restriction Endonuclease Digestion of Nuclear DNA

Digestion of D-550 nuclear DNA with Pst I was as described for restriction of v-fes DNA from pBR322 (steps 1-7). DNA was suspended in approximately 10 µl of 1X TE, pH 8.0 per 50 µg of DNA restricted.

Southern Transfer of Nuclear DNA

This procedure was used as described by Maniatis et al., 1982, pp. 382-386.

1) After electrophoresis in 1% agarose gels and staining with EtBr, the DNA was photographed using 254 nm UV light.

2) The DNA was denatured in several volumes of 1.5M NaCl and 0.5M NaOH for 1 hr at room temperature with constant agitation.
3) The gel was neutralized in several volumes of 1M Tris-HCl, pH 8.0 and 1.5M NaCl for 1 hr at room temperature with constant agitation.

4) GeneScreen membrane, the size of the gel, was prewetted with 2X SSC for 20 min.

5) The DNA was eluted from the gel onto the GeneScreen membrane in 10X SSC (1.5 M NaCl, 0.15 M Na citrate) for 20–24 hrs at 25°C.

6) The gel was discarded and the membrane was soaked in 6X SSC (0.9M NaCl, 0.09M Na citrate) at room temperature for 5 min. to remove residual agarose.

7) The filter was air dried at room temperature, and the DNA was fixed by heating the membrane for 2-4 hrs at 80-100°C.

8) DNA was hybridized to $^{32}$P-v-fes as described for the dot-blot procedure, using formamide without dextran sulfate as an enhancer (steps 5-10). Autoradiography of Southern transferred DNA after hybridization to $^{32}$P-v-fes was as described for the dot-blot procedure (steps 1-3).
RESULTS

Analysis of RNA Purified from KT-FeLV

KT-FeLV purified RNA sedimented in sucrose density gradients as two distinct species with sedimentation values of 35S and 70S and as a broad band of highly absorbing material at 254 nm with a sedimentation value of less than 20S (Figure 3).

The sedimentation pattern of purified KT-FeLV RNA was consistent with that expected for a 70S genome consisting of 35S dimer subunits and associated with small t-RNA and r-RNA molecules with a sedimentation value of less than 20S. The 35S RNA molecule was collected and used primarily as the RNA template in the C-DNA synthesis reaction. The 70S RNA molecule was also collected and denatured by heating in boiling water for 45 sec. to dissociate it into its 35S subunit molecules.

After heating, the 70S RNA sedimented in a broad zone in the top one-third of sucrose gradients in a sedimentation range of approximately 5-25S (Figure 4). This indicated that the 35S RNA species was not intact, but had dissociated into a broad range of smaller RNA molecules after heating of the 70S species. Further analyses using heat denaturation from 30 sec. to 1 min. produced similar results. The 70S RNA molecule showed no evidence of nuclease degradation or nicking in preparative isolation procedures in sucrose gradients. It sedimented at the 70S position when compared to the 16S and 23S RNA
markers from E. coli. The broad zone of RNA in the top one-third of sucrose gradients (Figure 3) was so highly absorbing at 254 nm that no distinguishing peaks could be detected. The RNA isolated from FL-74 cells cultured for 12 hr or 7 days was apparently degraded. Nicking was revealed only by heat denaturation, which dissociated the aggregate structure of the 70S RNA molecule. The 70S RNA isolated from 12 hr cultures of KT-FeLV showed a similar degree of degradation to that isolated from 7-day-old cultures when analyzed on sucrose density gradients. No substantial differences were noted in the character of the native and heat-denatured RNA profiles from KT-FeLV grown for either 7 days or 12 hrs other than a lower recovery of RNA from 12 hr cultures.

The degraded state of the 35S and 70S RNA species from 12 hr and 7-day-old cultures of KT-FeLV was also verified by treatment with glyoxal prior to electrophoresis in 1.2% agarose gels. The glyoxalation prevented secondary and tertiary structural bonding and permitted accurate determination of molecular weight. The 35S RNA species was defined in agarose gels, but a large degree of smearing was also detected, indicating that a degraded population of RNA molecules less than 35S was present. The 35S species from 12 hr cultures appeared to be less degraded than that from 7-day-old cultures. The 70S species from both 12 hr and 7-day-old cultures also electrophoresed as populations of degraded RNA molecules in agarose gels (Figure 5). The nicked regions within the 35S and 70S RNA molecules prevented continuous copying of the templates in the C-DNA reaction. However, both the 35S and 70S RNA species were
used as templates in the C-DNA reaction in efforts to recover a small population of C-DNA molecules of suitable length as hybridization probes.

Analysis of Complementary DNA Prepared to 35S and 70S KT-FeLV RNA

The C-DNAs recovered from both the 35S and 70S RNA templates were in the same size range regardless of whether the virus was grown for 12 hrs or 7 days. The glyoxal treatment used to denature the C-DNA for sizing in agarose gels provided evidence that the C-DNAs existed in a heterogeneous, small size range as would be expected for a heterogeneous RNA template. The C-DNAs from both culture times, and from either the 35S or 70S template RNA, migrated faster than the 561 base pair marker from a Hind III digest of Lambda DNA (Figure 6). The short incomplete copies of C-DNA prepared to KT-FeLV 35S or 70S RNA were unsuitable as hybridization probes and reflected the denatured state of the RNA molecules used as templates.

Purity of the Nuclear and Cytoplasmic DNA Recovered from Human Fibroblast D-550 Cells

Readings were taken at wavelengths of both 260 nm and 280 nm for spectrophotometric quantitation and determination of purity of human DNA. The readings at 260 nm were used to calculate the concentration of nucleic acid in the sample (an OD of 1.0 corresponded to approximately 50 µg/ml for double-stranded DNA).

The nuclear DNA was also quantitated by comparison of levels of EtBr fluorescence to that of known quantities of calf thymus DNA. This verified the quantitation as determined by OD_{260} reading and was
used to quantitate any of the nuclear DNA preparations whose $\text{OD}_{260}/\text{OD}_{280}$ ratios were less than 1.8, indicating possible contamination with protein or phenol. In general, the nuclear DNA obtained from chemically-treated and ST-FeSV-infected D-550 cells was quite pure as judged from $\text{OD}_{260}/\text{OD}_{280}$ ratios (Table 1). Of the 15 different nuclear DNA preparations, only 4 had $\text{OD}_{260}/\text{OD}_{280}$ ratios of less than 1.8 and these were all 1.7. Quantitation of these 4 samples by comparison of EtBr fluorescence with that of calf thymus DNA standards was similar to that obtained by $\text{OD}_{260}$ reading.
Table 1
Purity of D-550 DNA - OD\textsubscript{260}/OD\textsubscript{280} Ratios

<table>
<thead>
<tr>
<th>Time of Chemical Treatment Relative to ST-FeSV Infection</th>
<th>OD\textsubscript{260}/OD\textsubscript{280}</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Hydrazine, 2 hr Pre-</td>
<td>1.70</td>
</tr>
<tr>
<td>2) Hydrazine, 2 hr Post-</td>
<td>1.83</td>
</tr>
<tr>
<td>3) Mono-methyl Hydrazine, 2 hr Pre-</td>
<td>1.73</td>
</tr>
<tr>
<td>4) Mono-methyl Hydrazine, 2 hr Post-</td>
<td>1.70</td>
</tr>
<tr>
<td>5) Symmetrical Dimethyl Hydrazine (SDMH), 2 hr Pre-</td>
<td>1.88</td>
</tr>
<tr>
<td>6) Symmetrical Dimethyl Hydrazine (SDMH), 2 hr Post-</td>
<td>1.91</td>
</tr>
<tr>
<td>7) Unsymmetrical Dimethyl Hydrazine (UDMH), 2 hr Pre-</td>
<td>1.91</td>
</tr>
<tr>
<td>8) Unsymmetrical Dimethyl Hydrazine (UDMH), 2 hr Post-</td>
<td>1.98</td>
</tr>
<tr>
<td>9) Benzo[a]pyrene, 2 hr Pre-</td>
<td>1.70</td>
</tr>
<tr>
<td>10) Benzo[a]pyrene, 2 hr Post-</td>
<td>1.94</td>
</tr>
<tr>
<td>11) Pyrene, 2 hr Pre-</td>
<td>2.05</td>
</tr>
<tr>
<td>12) Pyrene, 2 hr Post-</td>
<td>1.97</td>
</tr>
<tr>
<td>13) Phenyl-α-Naphthylamine (PANA), 2 hr Post-</td>
<td>1.78</td>
</tr>
<tr>
<td>14) No Chemical Treatment, ST-FeSV only</td>
<td>1.94</td>
</tr>
<tr>
<td>15) No Chemical Treatment or Virus Infection,</td>
<td>Normal D-550 DNA</td>
</tr>
</tbody>
</table>
The quantity of cytoplasmic DNA was too low to be measured by spectrophotometry. Also, residual traces of Tween-40 and sodium deoxycholate contributed to absorbance at 260 nm in excess of that due to DNA. Approximately 0.1 to 0.3 μg of cytoplasmic DNA was recovered per 40-490 cm² tissue culture roller flasks (per chemical treatment) as quantitated by comparison of EtBr fluorescence with that of known calf thymus DNA standards. Since the purification regimen for cytoplasmic DNA was nearly identical to that for nuclear DNA, cytoplasmic DNA was of acceptable purity for nucleic acid hybridization studies.

### Purity of Plasmid and v-fes DNA

Plasmid pBR322 DNA, alone or containing the subcloned v-fes fragments, was relatively free from protein and RNA contamination after pronase treatment and isolation from two cycles of CsCl centrifugation (Figure 7). \( \text{OD}_{260}/\text{OD}_{280} \) ratios were always above 1.80.

Restriction endonuclease digestion of the \( S_L \) and \( S_R \) subclones of v-fes in pBR322, with Pst I, did not always result in totally pure preparations of v-fes DNA. The low molecular weight v-fes resulting from Pst I digestion was collected from 15-30% sucrose density gradients (Figure 8) and assayed for purity on 1% agarose gels (Figure 9).

Electrophoresis and staining of restricted v-fes DNA from sucrose gradients indicated that the DNA migrated as a single narrow band of approximately 500 base pair size. The remainder of the digested DNA (linear pBR322) also migrated as a single band. However, there was some indication that pBR322 DNA sequences were cleaved along with
v-fes. The pBR322 DNA restricted from the S<sub>L</sub> subclone ran at a slightly faster rate in 1% agarose gels than the linear DNA from a commercial preparation of pBR322 DNA (Bethesda Research Laboratories) and from <i>E. coli</i> D5410 supplied by Dr. J. Reeve (The Ohio State University, Columbus, OH). This indicated that the linear pBR322 DNA from the Pst I-digest was of lower molecular weight than native linear pBR322 DNA. Suspecting the possibility that the restricted v-fes fraction might contain some pBR322 DNA sequences, this v-fes fraction was radiolabelled with <sup>32</sup>P and hybridized to pure pBR322 DNA. Hybridization to approximately 6 µg of pBR322 DNA resulted in intense exposure on the autoradiograph.

It was apparent that the v-fes probe possessed some homology to pBR322 DNA which enhanced hybridization between the probe and the copy standards (which contained unrestricted v-fes DNA), making them unsuitable as standards for quantitating v-fes copy numbers.

**Radiolabelling of Plasmid and v-fes DNA**

Radiolabelling of plasmid or v-fes DNA with <sup>32</sup>P by nick-translation resulted in specific activities between 7.0 to 10.0 x 10<sup>7</sup> dpm/µg of DNA.

**Investigation of Homology Between pBR322 DNA and Various Other Sources of DNA**

Since the v-fes probe contained some pBR322 DNA sequences, it was necessary to determine whether homology existed between pBR322 and human fibroblast (D-550) DNA. pBR322 DNA (covalently closed and supercoiled) from <i>E. coli</i> strain D5410 was treated with RNase A and pronase and extracted with TE-saturated-phenol:chloroform:isoamyl
alcohol (25:24:1) after isolation from CsCl equilibrium density gradients. Plasmid DNA was labelled with $^{32}P$ by nick-translation and used as a hybridization probe with the following DNA preparations:

- Salmon sperm DNA (100 to 12.5 μg);
- Calf thymus DNA (100 to 12.5 μg);
- Human (D-550) normal fibroblast DNA (56 to 7 μg);
- E. coli DNA (100 to 12.5 μg); and
- pBR322 DNA (approximately 3 μg).

DNA was hybridized for approximately 48 hrs, and the X-ray film was exposed to the membrane for 24 hrs. Results showed no detectable homology between $^{32}P$-pBR322 and human DNA. There was also no homology with calf thymus, or salmon sperm DNA (Figure 10). All dilutions of E. coli DNA from 100 to 12.5 μg demonstrated detectable homology to pBR322 DNA on autoradiographs due to contaminating traces of E. coli nicked chromosomal DNA which equilibrated with pBR322 DNA in CsCl gradients. These results verified that extraneous pBR322 sequences in the $^{32}P$-v-fes probe would not create background problems when used in dot-blot hybridizations to human fibroblast DNA.

Attempts to Block Out Background Homology to pBR322 DNA in Prepared Copy Standards

The copy standards were prepared using predetermined amounts of v-fes DNA in pBR322 plus denatured salmon sperm DNA. Traces of pBR322 DNA in the $^{32}P$-v-fes probe contributed to background hybridization to the standards in excess of that specific for v-fes, making their use for enumeration of copy numbers of integrated v-fes inaccurate. An attempt was made to circumvent this problem by
prehybridizing the copy standards with pBR322 DNA to block out background homology prior to hybridization with the $^{32}\text{P-}\text{v-fes}$ probe.

After prehybridization overnight, the membranes which contained copy standards of DNA were hybridized for 24 hrs to a 100-fold excess of pBR322 above that present in the spotted copy standards. The membranes were then washed twice with 0.025M Na$_2$HPO$_4$/NaH$_2$PO$_4$, pH 6.5, to remove unhybridized pBR322. The copy standards were then hybridized with $^{32}\text{P-}\text{pBR322}$ (1 x $10^6$ dpm/ml hybridization buffer) for approximately 60 hrs at 42°C. Membranes were washed extensively and the X-ray film was exposed to the membranes for 45 hrs.

Blocking of background homology to pBR322 DNA was effective from 2 copies through 26 copies of v-fes in the copy standards. Background homology was not completely blocked in the copy standards containing 50 to 300 copies of v-fes.

Comparison of the Efficiency of Restricted and Unrestricted v-fes DNA as Hybridization Probes

Initial detection of v-fes homology in human DNA was with the unrestricted S$_L$ subclone of v-fes as a radiolabelled probe (v-fes was not restricted from pBR322 plasmid DNA). Its hybridization to human DNA was never in sufficient amounts to be detected by autoradiography.

When v-fes DNA was labelled with $^{32}\text{P}$ after enzymatic restriction from pBR322 DNA and purification in rate zonal sucrose density gradients, the probe was much more specific for the c-fes and v-fes sequences in human DNA infected with ST-FeSV. The two copies of
c-fes present in normal diploid human DNA were always readily detectable using this $^{32}$P-v-fes probe. On occasion, when v-fes was not recovered from sucrose gradients totally free from restricted pBR322 DNA (detectable by the presence of a second band in agarose gels), its efficiency as a probe for v-fes in human DNA decreased proportionally with its degree of contamination with pBR322 DNA.

**Experimental Use of Dextran Sulfate as a Hybridization Enhancer**

Dextran sulfate was used experimentally to enhance hybridization of unrestricted $^{32}$P-v-fes in pBR322 to human nuclear DNA.

Dextran sulfate was included in both the prehybridization and hybridization solutions as described in Materials and Methods. Nonspecific background binding of the labelled probe was a tremendous problem with this method. The $S_L$ subclone of v-fes unrestricted from pBR322 demonstrated no detectable hybridization to normal human DNA in the presence of dextran sulfate and was not specific in detecting the 2 copies of c-fes per diploid genome.

The use of both dextran sulfate and unrestricted v-fes DNA as the probe in hybridization mixtures was rejected for the screening of chemically-treated and ST-FeSV-infected human DNA for v-fes homology. The best hybridization results were obtained when v-fes was restricted from pBR322 vector DNA and purified prior to nick-translation and when dextran sulfate was excluded from the hybridization solutions.

**Screening of Nuclear DNA from Human (D-550) Fibroblasts for Homology to v-fes**

The number of copies of v-fes present in ST-FeSV-infected D-550 cells was determined using normal uninfected human (D-550) DNA which
contains 2 copies of c-fes per diploid genome as the standard of comparison. Nuclear DNA from five separate preparations of normal D-550 fibroblasts consistently hybridized to $^{32}$P-v-fes and was detectable by autoradiography when spotted at levels as low as 2.5 μg per 10 μl volume. When compared to normal, uninfected cells, D-550 cells, infected with ST-FeSV only possessed 2 copies of integrated proviral v-fes DNA in addition to the normal background of 2 copies of c-fes.

Quantitative comparisons of all nuclear DNA were made visually and copy numbers of v-fes were verified by soft laser-densitometry of autoradiograms. Attempts were made to quantitate the radioactivity bound to the membranes by cutting out individual spots after hybridization and counting in a liquid scintillation counter. This was not practical since the SSC buffers contributed to high backgrounds on the GeneScreen membranes which were not uniform nor easily removed by extensive washing. Membranes were routinely exposed to X-ray film for at least 7 days to detect low copy numbers of v-fes. By this time radioactivity was usually too low for accurate quantitation by counting. Visual and densitometric determinations of genome numbers by comparison of densities of hybridized dilutions of DNA on X-ray film was usually adequate for copy numbers of 8 or less. All comparisons were made to a standard dilution series of normal D-550 nuclear DNA.

The type of buffer system used to pretreat the membrane and spot the nuclear DNA was crucial to the success of the dot-blot hybridization reactions and subsequent quantitation of genome copy numbers.
The high molecular weight, nuclear DNA did not bind well unless the membrane was pretreated with 2X or 10X SSC, and unless the DNA was spotted in 5X SSC to 10X SSC. Efficiency of binding of nuclear DNA in TE buffer was generally less than or equal to half that concentration of DNA from the same chemical treatment spotted in 5X SSC (Figure 11).

The dot-blot hybridization results from the nuclear DNA of human D-550 cells exposed to various chemicals at 2 hrs either before or after ST-FeSV infection are shown in Table 2 (data obtained from Figs. 12, 13, 16 and 17). The level of homology to v-fes includes the normal 2 copies of c-fes present in background DNA.
Table 2  
Comparison of Three Methods of Determining Copy Numbers of v-fes in Nuclear D-550 DNA

<table>
<thead>
<tr>
<th>Treatment (Relative to ST-FeSV infection)</th>
<th>Visual Observation (per genome)</th>
<th>Radioisotope Counting (per genome)</th>
<th>Soft-Laser Densitometry (per genome)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) None - Normal diploid DNA</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>2) ST-FeSV - infected only</td>
<td>4</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>3) Hydrazine, - 2 hr Pre-</td>
<td>2-4</td>
<td>3-4</td>
<td>2-3</td>
</tr>
<tr>
<td>4) Hydrazine, + 2 hr Post-</td>
<td>2-4</td>
<td>4</td>
<td>2-3</td>
</tr>
<tr>
<td>5) Mono-Methyl Hydrazine, - 2 hr Pre-</td>
<td>2-4</td>
<td>3-4</td>
<td>3-4</td>
</tr>
<tr>
<td>6) Mono-Methyl Hydrazine, + 2 hr Post-</td>
<td>4</td>
<td>5-6</td>
<td>3-4</td>
</tr>
<tr>
<td>7) SDMH, - 2 hr Pre-</td>
<td>4</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>8) SDMH, + 2 hr Post-</td>
<td>4</td>
<td>3-4</td>
<td>3</td>
</tr>
<tr>
<td>9) UDMH, - 2 hr Pre-</td>
<td>4</td>
<td>5</td>
<td>3-4</td>
</tr>
<tr>
<td>10) UDMH, + 2 hr Post-</td>
<td>4</td>
<td>4-5</td>
<td>3</td>
</tr>
<tr>
<td>11) Benzo[a]pyrene - 2 hr Pre-</td>
<td>8</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>12) Benzo[a]pyrene + 2 hr Post-</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>13) Pyrene, - 2 hr Pre-</td>
<td>4</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>14) Pyrene, + 2 hr Post-</td>
<td>8</td>
<td>6-7</td>
<td>5</td>
</tr>
<tr>
<td>15) PANA, + 2 hr Post-</td>
<td>4</td>
<td>4</td>
<td>2</td>
</tr>
</tbody>
</table>
Lack of a consistent pattern indicated that treatment with chemical carcinogens at 2 hrs after ST-FeSV infection did not generally inhibit v-fes integration coordinate with inhibition of transformation. Only pretreatment with benzo[a]pyrene and posttreatment with pyrene relative to ST-FeSV infection enhanced v-fes integration. This occurred at levels twice that observed with ST-FeSV-infected cells only. Only benzo[a]pyrene caused a 4-fold decrease in integrated v-fes copies in D-550 cells when the chemical was added after infection rather than before. Conversely, D-550 cells treated with the non-carcinogen pyrene demonstrated a 2-fold increase in v-fes integration when the chemical was added 2 hrs after versus 2 hrs previous to ST-FeSV infection. Treatment with mono-methyl hydrazine or PANA at 2 hrs after ST-FeSV infection or treatment with SDMH or UDMH at either 2 hrs before or 2 hrs after virus infection did not alter integration of v-fes relative to that in ST-FeSV-infected cells. In some hybridizations, DNA from cells treated with hydrazine at both 2 hrs before or after ST-FeSV infection demonstrated 2-fold fewer copy numbers of integrated v-fes than DNA from ST-FeSV-infected cells only. Similarly, cells treated with mono-methyl hydrazine at 2 hrs before infection with ST-FeSV showed equal or 2-fold fewer numbers of integrated v-fes relative to virus-infected cells only.

Screening of Cytoplasmic DNA from Human (D-550) Fibroblasts for Homology to v-fes

Cytoplasmic DNA from D-550 cells infected with ST-FeSV and treated with chemicals at 2 hrs before or after infection was
screened for homology to v-fes. No homology was detected in any of the cytoplasmic DNA preparations from chemically-treated and virus-infected cells or from cells infected with ST-FeSV only, when cells were harvested at 6 days after infection in order to duplicate the experimental conditions used in the ST-FeSV - D-550 assays for detecting chemical carcinogens (Blakeslee et al., 1983a; 1983b; 1983c). The nuclear DNA hybridization studies suggested that the proviral DNA had integrated by this time, indicating that provirus or v-fes synthesis and migration into the nucleus was not affected by chemicals. Transformed foci were generally also present by this time.

Homology to v-fes was never detected in autoradiographs when up to 10 to 30 ng of cytoplasmic DNA were spotted per 10 μl volume. In contrast, control v-fes DNA showed intense hybridization when as little as 0.625 μg was spotted on membranes and when films were exposed for as short a time as 12 hrs (Figures 14 and 15).

Detection of Homology to v-fes in Human D-550 DNA After Southern Blot Transfer

It was of interest to determine whether v-fes integrated into D-550 DNA as a unique sequence or as part of a larger sequence of proviral DNA, since human cells are non-permissive hosts for ST-FeSV. Normal D-550 DNA, DNA from cells infected with ST-FeSV only, and DNA from D-550 cells treated with hydrazine or benzo[a]pyrene at 2 hrs after infection with ST-FeSV were digested with restriction endonuclease, Pst I. Each of these digested preparations electrophoresed as a continuous population of many molecular weight size classes from
greater than 4,000 base pairs to less than 500 base pairs in agarose gels. No distinct bands were visible. When this DNA was eluted onto GeneScreen membranes by Southern transfer and hybridized to \textit{v-fes}, a similar pattern of hybridized DNA was observed on autoradiographs. Sonication of DNA to about 2,000 base pairs and \textit{Pst I} digestion resulted in a continuous molecular weight range of DNA molecules which hybridized to \textit{v-fes} because of homology to \textit{c-fes} in the normal background of human DNA. However, it was anticipated that autoradiographs would show a more intense DNA band with homology to \textit{v-fes} at about 500 base pairs in all but normal DNA if \textit{v-fes} was integrated as part of the total proviral sequence. \textit{Pst I} cleaves near both ends of the \textit{v-fes} gene and near its center to produce two \textit{v-fes} fragments of approximately 500 base pairs in length. If only partial segments of \textit{v-fes} were integrated, a distinct band would not occur at 500 base pairs. No 500 base pair fragment with \textit{v-fes} homology above background was observed in Southern blots of DNA from chemically-treated and/or \textit{ST-FeSV}-infected D-550 cells.
Figure 3.

Profile of KT-FeLV RNA from 12 hr or 7-day-old cultures after sedimentation in sucrose density gradients. The RNA fractionated in three distinct regions representing the 35S subunit, the 70S dimer, and a region of low molecular weight RNA species less than 20S. Marker ribosomal RNA from E. coli was subjected to identical sedimentation analyses. It sedimented as a 16S species, a 23S species, and a 39S aggregate. Purified RNA from KT-FeLV or E. coli was layered onto 15-30% linear sucrose gradients prepared in 1X TNE, pH 7.4 and centrifuged at 38,000 RPM for 3.75 hrs in a Beckman SW41 rotor at 25°C.
Figure 3

FeLV-KT RNA

E. coli Markers

Figure 3
Figure 4.

Profiles of total KT-FeLV RNA and heated 70S RNA after sedimentation in sucrose density gradients. KT-FeLV 70S RNA was isolated from total viral RNA and heated for 45 sec. in boiling water. The heated RNA was centrifuged in 15-30% linear sucrose density gradients for 3.75 hrs at 38,000 RPM in a Beckman SW41 rotor at 25°C. Similar sedimentation patterns resulted when 70S KT-FeLV RNA from 12 hr or 7-day-old cultures was heated.
Figure 4

FeLV-KT RNA

Absorbance 254 nm

Top Bottom

35S 70S

Heated 70S FeLV-RNA

Absorbance 254 nm

Top Bottom
Figure 5.

Glyoxalated and non-glyoxalated KT-FeLV RNA after electrophoresis for 2 hrs at 45 mAmps in 1.2% agarose and staining with acridine orange.

Lane 1: 2.25 µg 35S RNA (plus glyoxal) from 12 hr cultures.
Lane 2: 2.25 µg 35S RNA (no glyoxal) from 12 hr cultures.
Lane 3: 2.25 µg 35S RNA (plus glyoxal) from 7-day-old cultures.
Lane 4: 2.25 µg 35S RNA (no glyoxal) from 7-day-old cultures.
Lane 5: 4.0 µg E. coli 16S and 23S r-RNA markers (plus glyoxal).
Lane 6: 4.0 µg E. coli 16S and 23S r-RNA markers (no glyoxal).
Lane 7: 1.25 µg 70S RNA (plus glyoxal) from 12 hr cultures.
Lane 8: 1.25 µg 70S RNA (no glyoxal) from 12 hr cultures.
Lane 9: 2.0 µg 70S RNA (plus glyoxal) from 7-day-old cultures.
Lane 10: 2.0 µg 70S RNA (plus glyoxal) from 7-day-old cultures.
Figure 5
Figure 6.

Glyoxalated and non-glyoxalated Complementary DNA prepared to KT-FeLV RNA after electrophoresis for 2 hrs at 45 mAmperes in 1.2% agarose and staining with acridine orange.

Lane 2: 1 μg C-DNA prepared to 35S RNA from 12 hr cultures (plus glyoxal).
Lane 3: 1 μg C-DNA prepared to 35S RNA from 12 hr cultures (no glyoxal).
Lane 4: 1 μg C-DNA prepared to 35S RNA from 7-day-old cultures (plus glyoxal).
Lane 5: 1 μg C-DNA prepared to 35S RNA from 7-day-old cultures (no glyoxal).
Lane 6: 3.36 μg marker DNA (plus glyoxal). Markers were generated by digestion of Lambda DNA with Hind III (Boehringer Mannheim). Marker fragments consisted of 23,606; 9,636; 6,636; 4,333; 2,257; 1,985; 561; and 139 base pairs. (Note degradation of commercial preparation.)
Lane 7: 3.36 μg marker DNA (no glyoxal). Same fragments present as in lane 5.
Lane 8: 1 μg C-DNA prepared to 70S RNA from 7-day-old cultures (plus glyoxal).
Lane 9: 1 μg C-DNA prepared to 70S RNA from 7-day-old cultures (no glyoxal).
Lane 10: C-DNA to 70S RNA from 7-day-old cultures after gel filtration on Sephadex (plus glyoxal).
Lane 11: C-DNA to 70S RNA from 7-day-old cultures after gel filtration on Sephadex (no glyoxal).
Figure 7

pBR322 DNA containing the $S_L$ subclone of $v$-fes after a second cycle of centrifugation in CsCl equilibrium density gradients. Centrifugation was for 48 hrs at 41,000 RPM in the SW50.1 rotor of a Beckman Model L8-80 ultracentrifuge at 15°C. The upper band represents relaxed linear, bacterial DNA and nicked circular, plasmid DNA which equilibrated at a density of 1.55 gm/ml. The bottom band indicated by the arrow represents covalently closed superhelical plasmid DNA which equilibrated at 1.59 gm/ml and which was collected for restriction of $v$-fes.
Figure 8

Restricted v-fes DNA in 15-30% sucrose density gradients after digestion of the $S_L$ subclone in pBR322 with Pst I. Centrifugation was for 5.25 hrs at 15°C in the SW50.1 rotor of a Beckman Model L8-80 ultracentrifuge at 40,000 RPM. v-fes DNA, indicated by the arrow was collected and radiolabelled with $^{32}\text{P}$ for use as a hybridization probe. The dense band below v-fes DNA represents linear pBR322 DNA remaining after v-fes restriction.
Figure 9.

Electrophoresis of v-fes and pBR322 plasmid DNA in 1% agarose gels. Electrophoresis was for 1.5 hrs in 10 mM Na phosphate buffer, pH 7.0 at 45 mAmps constant current and staining was with acridine orange.

Lane 3: represents Pst I restricted v-fes DNA collected from sucrose density gradients. The top band is contaminating linear pBR322 DNA from which the S_L subclone of v-fes was restricted and the lower band is v-fes DNA.

Lane 4: represents linear pBR322 DNA resulting from Pst I restriction of the S_L subclone of v-fes.

Lane 5: represents unrestricted pBR322 DNA containing the S_L subclone of v-fes. The top band is linear plasmid DNA and the bottom band is covalently closed, supercoiled DNA.

Lane 6: represents a commercial preparation of pBR322 DNA (Bethesda Research Laboratories, Inc.). The top band is open circle and the bottom band is linear DNA.

Lane 7: represents pBR322 DNA from E. coli strain D5410. The top band is open circle, the middle band is linear, and the bottom band is covalently closed, supercoiled DNA.

Lane 8: represents DNA markers generated by Hind III digestion of Lambda DNA. The six visible bands represent 23,606; 9,636; 6,636; 4,333; 2,257; and 1,985 base pairs. The 561 and 139 base pair markers are not visible.
Figure 10.

Dot-blot hybridization between $^{32}$P-labelled pBR322 DNA and DNA from salmon sperm, calf thymus, human fibroblast (D-550) cells, E. coli, and pBR322 plasmid. All DNA was spotted in 10 ul volumes.

Lane 1: represents pBR322 DNA spotted in duplicate amounts of 3 ug in rows A and B.

Lane 2: represents E. coli DNA spotted in doubling dilutions of 100 pg to 12.5 pg in rows A through D, respectively.

Lane 3: represents normal DNA from human skin fibroblast cells (D-550) spotted in doubling dilutions from 56 to 7 pg in rows A through D, respectively.

Lane 4: represents calf thymus DNA spotted in doubling dilutions from 100 to 12.5 pg in rows A through D, respectively.

Lane 5: represents salmon sperm DNA spotted in doubling dilutions from 100 to 12.5 pg in rows A through D, respectively.

Hybridization solutions included formamide as described in Materials and Methods.
Figure 11.

Variability of DNA binding as a function of buffer as shown by dot-blot hybridizations between $^{32}$P-labelled v-fes and nuclear DNA from human (D-550) fibroblasts. The GeneScreen membrane was pretreated with 2X SSC. Row A represents DNA spotted in 1X TE Buffer, pH 8.0. Rows B through E represent DNA spotted in 5X SSC buffer. DNA from various chemical treatments and/or infected with ST-FeSV was spotted in doubling dilutions of 10 µl volumes as follows:

Lane 1: Hydrazine, - 2 hr before ST-FeSV infection. 20 - 1.25 µg in rows A-E, respectively.
Lane 2: Hydrazine, + 2 hr after ST-FeSV infection. 40 - 2.50 µg in rows A-E, respectively.
Lane 3: Mono-methyl hydrazine, - 2 hr before ST-FeSV infection. 20 - 1.25 µg in rows A-E, respectively.
Lane 4: Mono-methyl hydrazine, + 2 hr after ST-FeSV infection. 40 - 2.5 µg in rows A-E, respectively.
Lanes 5 and 6: ST-FeSV infection only. 30 µg in row A. 20 - 1.25 µg in rows B-E, respectively.
Lane 7: SDMH, - 2 hr before ST-FeSV infection. 40 - 2.5 µg in rows A-E, respectively.
Lane 8: SDMH, + 2 hr after ST-FeSV infection. 40 - 2.5 µg in rows A-E, respectively.
Lane 9: Normal, uninfected D-550 DNA. 40 - 2.5 µg in rows A-E, respectively.
Figure 12.

Dot-blot hybridization of $^{32}\text{P-v-fes}$ to nuclear DNA from human fibroblast (D-550) cells treated with chemicals and/or infected with ST-FeSV. The membrane was treated with 10X SSC and DNA was spotted in doubling dilution series in 10 μl volumes. Rows A-E represent 40, 20, 10, 5 and 2.5 μg of DNA, respectively. DNA was from the following treated and untreated cells:

Lane 1: UDMH, - 2 hr before ST-FeSV infection.
Lane 2: UDMH, + 2 hr after ST-FeSV infection.
Lane 3: Pyrene, - 2 hr before ST-FeSV infection.
Lane 4: Pyrene, + 2 hr after ST-FeSV infection. (40 μg sample not done.)
Lane 5: Normal, uninfected DNA.
Lane 6: ST-FeSV infected DNA only. (40 μg sample not done.)
Lane 7: Benzo[a]pyrene, - 2 hr before ST-FeSV infection. (40 μg sample not done.)
Lane 8: Benzo[a]pyrene, + 2 hr after ST-FeSV infection.
Lane 9: PANA, + 2 hr after ST FeSV infection.

Genome copy numbers were determined by comparison of densities of hybridized DNA blots to that of normal, uninfected DNA (see Table 2).
Figure 13.

Dot-blot hybridization of $^{32}$P-v-fes to nuclear DNA from human fibroblast (D-550) cells treated with chemicals and/or infected with ST-FeSV. The membrane was treated with 10X SSC and DNA was spotted in doubling dilution series in 10 μl volumes. Rows A-E represent 40, 20, 10, 5, and 2.5 μg of DNA, respectively. DNA was from the following treated and untreated cells:

- **Lane 1**: Hydrazine, - 2 hr before ST-FeSV infection.
- **Lane 2**: Hydrazine, + 2 hr after ST-FeSV infection.
- **Lane 3**: Mono-methyl hydrazine, - 2 hr before ST-FeSV infection.
  (40 μg sample not done.)
- **Lane 4**: Mono-methyl hydrazine, + 2 hr after ST-FeSV infection.
- **Lane 5**: Normal, uninfected DNA.
- **Lane 6**: ST-FeSV-infected DNA only. (40 μg sample not done.)
- **Lane 7**: SDMH, - 2 hr before ST-FeSV infection.
- **Lane 8**: SDMH, + 2 hr after ST-FeSV infection.

Genome copy numbers were determined by comparison of densities of hybridized DNA blots to that of normal, uninfected DNA (see Table 2).
Figure 14.

Dot-blot hybridization of $^{32}$P-v-fes to cytoplasmic DNA from human fibroblast (D-550) cells treated with chemicals and/or infected with ST-FeSV. DNA was spotted in doubling dilution series in 10 µl volumes. Lane 8 represents restricted v-fes DNA from the $S_L$ subclone spotted at 50 - 3.125 ng in rows A-E, respectively. DNA was spotted from 10 - 0.625 ng in rows A-E, respectively for lanes 1-7.

DNA was from the following treated or untreated cells:

Lane 1: Normal, uninfected DNA.
Lane 2: ST-FeSV infected DNA only.
Lane 3: Pyrene, - 2 hr before ST-FeSV infection.
Lane 4: Pyrene, + 2 hr after ST-FeSV infection.
Lane 5: Benzo[a]pyrene, - 2 hr before ST-FeSV infection.
Lane 6: Benzo[a]pyrene, + 2 hr after ST-FeSV infection.
Lane 7: PANA, + 2 hr after ST-FeSV infection.
Figure 15.

Dot-blot hybridization of $^{32}$P-v-fes to cytoplasmic DNA from human fibroblast (D-550) cells treated with chemicals and/or infected with ST-FeSV. DNA was spotted in doubling dilution series in 10 μl volumes. Lane 9 represents restricted v-fes DNA from the $S_L$ subclone spotted at 10 - 0.625 ng in rows A-E, respectively. DNA was spotted from 30 - 1.875 ng in rows A-E, respectively for lanes 1-8.

DNA was from the following treated cells:

Lane 1: Mono-methyl hydrazine, - 2 hr before ST-FeSV infection.
Lane 2: Mono-methyl hydrazine, + 2 hr after ST-FeSV infection.
Lane 3: UDMH, + 2 hr after ST-FeSV infection.
Lane 4: ST-FeSV-infected DNA only.
Lane 5: SDMH, - 2 hr before ST-FeSV infection.
Lane 6: SDMH, + 2 hr after ST-FeSV infection.
Lane 7: Hydrazine, - 2 hr before ST-FeSV infection.
Lane 8: Hydrazine, + 2 hr after ST-FeSV infection.
Figure 16.

Soft-laser densitometry scan of autoradiograph of membrane containing $^{32}$P-v-fes hybridized to nuclear DNA from human fibroblast (D-550) cells treated with chemicals and/or infected with ST-FeSV. The chemical treatment and time of addition (in hrs) of each chemical relative to ST-FeSV infection is denoted above each peak. Relative intensities of integrated areas under each peak on the curve were compared to ST-FeSV-infected cells only and are given directly above each peak. Genome copy numbers were calculated based on normal human DNA containing 2 copies of c-fes per diploid genome (Table 2).
Figure 16

- PANA, +2
- BP, +2
- BP, -2
- FeSV only
- Normal
- Pyrene, +2
- Pyrene, -2
- UDMH, +2
- UDMH, -2
Figure 17.

Soft-laser densitometry scan of autoradiograph of membrane containing $^{32}$P-v-fes hybridized to nuclear DNA from human fibroblast (D-550) cells treated with chemicals and/or infected with ST-FeSV. The chemical treatment and time of addition (in hrs) of each chemical relative to ST-FeSV infection is denoted above each peak. Relative intensities of integrated areas under each peak on the curve were compared to ST-FeSV-infected cells and are given directly above each peak. Genome copy numbers were calculated based on normal human DNA containing 2 copies of c-fes per diploid genome (Table 2).
DISCUSSION

Retroviruses possess a 60-70S single-stranded RNA genome which exists as a dimer of two genetically identical RNA subunit molecules sedimenting at 28-35S (Wang, 1978; Varmus, 1982). The functional significance of the dimer is unknown, and both subunits function in replication. A region of hydrogen-bonding occurs near the 5' end of each subunit molecule holding them together, but its precise location and structure are not absolutely known. KT-FeLV RNA purified from FL-74 cells sedimented as typical retrovirus 35S and 70S species. A zone of low molecular weight RNA species with sedimentation values of less than 20S was also observed (Figure 3). Small molecules normally associated with retrovirus RNA include specific t-RNAs (one molecule of which associates with each dimer subunit and primes the viral RNA-dependent DNA polymerase during replication synthesis of minus proviral DNA), cellular 4S t-RNAs which are carried along with the virus as it buds from the cell, and various r-RNAs (5S, 18S, and 28S) which are cellular in origin (Canaani et al., 1973; Cheung et al., 1972; Duesberg and Vogt, 1973; Erikson, 1969; Jarrett et al., 1971; Larsen et al., 1973; and Maizel et al., 1977).

Other workers analyzed viral RNA from various strains of FeLV. Brian et al. (1975) analyzed Rickard-FeLV RNA produced by a permanently infected feline thymus tumor line designated F-422. They identified three size classes of RNA by gel electrophoresis. These
included: 1) a $6.2 \times 10^6$ to $7.1 \times 10^6$ d mol. wgt. class which
sedimented at 50-60S and comprised 57 to 76% of the total viral RNA;
2) a $8.7 \times 10^4$ d mol. wgt. class which corresponded to the 8S RNA
reported in murine sarcoma virus (Emanoil-Rabinovitch et al., 1973;
Larsen et al., 1973); and 3) a $2.5 \times 10^4$ d mol. wgt. class which
sedimented with 4S cellular t-RNA and comprised 6 to 12% of the total
viral RNA. They assumed that one 50 to 60S molecule existed per
virion. Based on that assumption, there were 2 to 7 8S molecules and
20 to 60 four to 5S molecules per virion. Brian et al. (1975)
concluded that the 50 to 60S RNA of Rickard-FeLV consisted of as few
as two $3.2 \times 10^6$ mol. wgt. subunits, or as many as four $1.8 \times 10^6$
mol. wgt. subunits.

Kimball and Rea (1980) analyzed the RNA from Gardner-Arnstein
(GA)- and ST-feline leukemia and sarcoma virus mixtures. The RNA
from the ST-FeLV-FeSV complex sedimented in a double-peaked band
ranging in size from 50-70S whereas GA-FeLV-FeSV RNA sedimented as a
single 70S peak. ST-FeLV RNA sedimented as a single 70S band, in
contrast to Rickard-FeLV RNA which sedimented more slowly at 50-60S.
Methods generally used to dissociate retrovirus-aggregate RNA
crystals into their separate subunits include heating in boiling
water, or treatments with urea, formamide, formaldehyde or DMSO
(Bader and Steck, 1969; Collett and Faras, 1977; Duesberg, 1970;
Kimball and Rea, 1980). After using thermal denaturation to reveal
subunit classes of RNA, Kimball and Rea (1980) found that three
subunit classes existed in ST-FeLV-FeSV RNA: 35-37S, 32-34S, and 25S.
ST-FeLV possessed the 35-37S subunit. The 32-34S class was similar
to subunits in Rickard-FeLV RNA and minor components of GA-FeLV-FeSV. The 25S subunit class was detected only in ST-FeLV-FeSV RNA.

KT-FeLV possessed a 70S species similar to GA-FeLV and ST-FeLV. The 35S subunit detected in KT-FeLV RNA appeared to be most similar in size to the 35-37S subunit of ST-FeLV (Figure 3). The 32-34S RNA subunit found by Kimball and Rea (1980) in ST-FeLV-FeSV and Rickard-FeLV was not present in KT-FeLV RNA purified by proteinase K digestion, phenol extraction, and sucrose density gradient centrifugation. Since differences exist among the RNA molecules of GA-FeLV, ST-FeLV, and Rickard-FeLV, KT-FeLV RNA may also differ from other FeLV strains by possessing a large proportion of low mol. wgt. molecules (less than 20S) which contribute to the aggregate-RNA 70S genome.

The culture age of FeLV at the time of purification is a critical determinant of viral RNA size. Brian et al. (1975) noted variation in the characteristics of Rickard-FeLV with age. The electrophoretic mobility of the 50 to 60S molecule was variable depending upon the length of time the virus was grown. High mol. wgt. RNA of $7.1 \times 10^6$ d was recovered from virus harvested after 20 hrs, whereas RNA from virus harvested at 4 hrs had an apparent mol. wgt. of $6.4 \times 10^6$ d. Similar observations were made by East et al. (1973b) in that they reported sedimentation coefficients of 58S and 50S for RNA from Rickard-FeLV grown for 20 hrs and 2 hrs, respectively.

Several workers have attempted to explain such discrepancies on the basis of a maturational process in which the RNA undergoes modification in the virion after budding, causing it to sediment at a
faster rate (Canaani et al., 1973; Cheung et al., 1972; East et al., 1973a; East et al., 1973b). Immature Rous sarcoma virus (3 to 60 minutes old) contains subunits ranging in size from 15 to 60S which apparently then assemble into the larger 68S molecule (Cheung et al., 1972). Assembly of the 58S molecule in murine sarcoma virus (East et al., 1973a) and in FeLV (East et al., 1973b) was suggested to occur from two or more 50S molecules. Brian et al. (1975) postulated that assembly of the 60S RNA molecule of Rickard-FeLV apparently occurred between 2 or more 50S species or between a 50S and a smaller (4S to 8S) molecule. They also discovered that denatured high mol. wgt. subunits from 20 hr-old FeLV RNA electrophoresed and sedimented in a more heterogeneous pattern than the same RNA species from 4 hr-old virus. Brian et al. (1975) postulated that nicks contained within the loops of an aggregate molecule (20 hr) allowed for secondary structural changes (perhaps ends of loops became more tightly folded) thereby creating a slower electrophoretically migrating or faster sedimenting molecule compared to the unnicked aggregate (4 hr). Sixty S to 70S molecules of RSV RNA apparently retained their aggregate structure in the presence of nicks which were revealed only after the aggregate was denatured (Bader, 1970).

The age of KT-FeLV at time of purification is also critical in obtaining RNA free from nicking due to nucleases (Paul Kimball, Battelle Memorial Institute, Columbus, OH - personal communication). The 70S molecule of KT-FeLV RNA grown for 12 hrs or 7 days was apparently nicked, but nicking was not obvious in sucrose density sedimentation analyses until the molecule was heat denatured (Figure
4). In addition, removal of secondary structure by glyoxalation revealed a degraded population of RNA molecules after electrophoresis of the 35S or 70S species in agarose gels (Figure 5). Apparently degradation of the RNA from most strains of FeLV is common in virus harvested after 3 hrs (Paul Kimball, Battelle Memorial Institute, Columbus, OH; Leland Velicer, Michigan State University, East Lansing, MI - personal communications). KT-FeLV virus grown for more than 3 hrs possessed RNA unsuitable for preparation of full-length C-DNA copies in that nicks interfered with proviral DNA synthesis in vitro (Figure 6).

In addition, the nature of even full length C-DNA prepared to FeLV RNA prevents it from being specific for that portion of the FeSV genome responsible for transformation. Approximately only 35.90% of the ST-FeLV genome is homologous to ST-FeSV RNA (Sherr et al., 1980). The degree of homology between KT-FeLV and ST-FeSV is less well known. Homologous regions of ST-FeLV and ST-FeSV RNA include the 5' end of the gag gene which codes for group-specific antigens, a portion of the 5' end of the envelope (env) gene, and the small c or common region of the 3' end which is present in normal cat cellular DNA (Figures 18 and 19). The pol region which codes for reverse transcriptase is not present in ST-FeSV and the unique v-fes or transforming gene function of ST-FeSV has no counterpart in the helper leukemia virus.

It is generally accepted that proviral DNA synthesis is initiated approximately 100 base pairs from the 3' end of the 5' ETR (or LTR- long terminal repeat) on the RNA template and then jumps to
the 3' LTR before proceeding with the synthesis of the minus DNA proviral strand in a 3' to 5' direction (Varmus, 1982). The reaction conditions for C-DNA synthesis in vitro produced a single-stranded copy which was not homologous to the full length of the KT-FeLV RNA genome due to nicking within the RNA molecule. The first regions copied presumably included a small region of the 5' end of the gag gene, the c region, and possibly a small portion of the env gene (Varmus, 1982). Furthermore, all of these regions were present in the helper FeLV proviral DNA which integrated in addition to FeSV proviral DNA during virus infection.

Because of difficulties in obtaining sufficient undegraded FeLV RNA for full-length C-DNA synthesis and due to the nonspecificity of a C-DNA probe to FeLV RNA for ST-FeSV, it was more practical to probe for ST-FeSV proviral DNA with a v-fes probe cloned in pBR322 plasmid. Since chemical carcinogens primarily altered transformation of D-550 cells when added at various times relative to ST-FeSV infection, it was advantageous to use a probe specific for the transforming function (encoded by v-fes) of the virus.

Various methodologies were explored in efforts to develop the best procedure for screening chemically-treated and/or ST-FeSV-infected D-550 cells for quantitation of v-fes integration. Prior to the discovery that human DNA contains c-fes, copy number standards of v-fes in pBR322 plus salmon sperm DNA were used for quantitation of v-fes. Attempts to block out background homology to pBR322 in copy standards which hybridized to similar sequences in the $^{32}$P-labelled
probe were unsuccessful beyond 26 copies of v-fes per diploid genome (Results, p. 94).

Blocking of pBR322 DNA in the copy standards may have been less efficient than expected for several reasons. Prehybridization to pBR322 may not have included a sufficient excess of pBR322 DNA to block all sites of homology in the copy standards. Washing of the membranes after prehybridization to unlabelled pBR322 DNA may also have been inadequate. This may have allowed unlabelled pBR322 DNA, which had only partially annealed to the copy standards or which had nonspecifically adsorbed to the membrane to hybridize readily to \(^{32}\)P-pBR322 DNA. Attempts to perfect blocking of background homology to pBR322 in copy standards were no longer pursued when it was determined that normal D-550 diploid DNA, which contains two copies of c-fes per genome, could be used as the standard for quantitation.

Detection of low copy numbers of v-fes in chemically-treated and/or ST-FeSV-infected and normal human DNA required that the \(^{32}\)P-v-fes probe be as free of contaminating pBR322 DNA as possible to increase the specificity of hybridization (Results, p. 95). It was reported by Rodland and Russell (1983) that a critical element in the success of hybridization reactions using DNA immobilized on GeneScreen membranes is the purity and specificity of the labelled probe. Their best results were obtained when the probe fragment of interest was separated from vector DNA by digestion with appropriate endonucleases and preparative electrophoresis. Rate zonal sucrose density gradient centrifugation was used as opposed to electrophoresis for final purification of v-fes from pBR322 (Figure 8) to
avoid contaminating compounds (sulfonated carbohydrates) in agarose which often interact with and impair the efficacy of enzymes used in nick-translation (Davis et al., 1980).

Dextran sulfate was used experimentally in an attempt to enhance hybridization of unrestricted $^{32}$P-v-fes in pBR322 to human nuclear DNA (Results, p. 96). This approach would have been useful because restriction endonuclease digestion of v-fes from pBR322 and additional purification of v-fes would not have been necessary. More DNA would also have been available for use in nick-translation. However, the unrestricted probe showed no detectable hybridization to normal human DNA in the presence of dextran sulfate. The mass of the labelled probe is critical in eliminating background when using dextran sulfate (GeneScreen Hybridization Transfer Membrane, Catalog No. NEF-972, Instruction Manual, New England Nuclear, 1982, p. 6). It was necessary to use twice the recommended mass per volume of labelled probe in order to provide sufficient label for detection of low copy numbers of v-fes. This may have contributed to problems with background binding of labelled probe to the membrane.

Three different methods were used to analyze the dot-blot hybridizations of $^{32}$P-v-fes to nuclear DNA and to quantitate relative genome copy numbers of v-fes (Table 2). Primary analyses were based upon semi-quantitative visual comparisons of autoradiographs from which the intensities of hybridized DNA were compared after several different exposure times. Visual comparison of autoradiographs was supplemented with analyses by radioisotope counting of hybridized DNA and soft-laser densitometry of autoradiographs. Background binding
of $^{32}$P-v-fes probe to the membrane, which could not totally be removed by washing, sometimes contributed to inaccurate interpretation from liquid scintillation counting which was not detected without simultaneous comparison of autoradiographs. Determination of genome copy number by densitometry did not always correlate with visual and radioisotope counting analyses due primarily to the nature of the technique. Laser scanning was done as a series of bands throughout the dot. This sometimes was interpreted by the computer as more than one peak in cases in which the DNA did not spread evenly over the membrane surface. In such situations, certain peaks were removed for ease in computer analyses. This may have contributed to discrepancies in densitometry analyses relative to visual comparisons of autoradiographs and counting of spotted DNA on the membrane. Regardless of which method was used to determine the copy number of v-fes per genome, all chemical treatments for that method of analysis were calculated on the basis of normal human DNA being equal to 2 copies per diploid genome.

Of 13 different chemical treatments, only three definitely appeared to consistently alter ST-FeSV, v-fes integration in D-550 cells, relative to cells infected with ST-FeSV only (Table 2). These included pre- and posttreatment with benzo[a]pyrene and posttreatment with pyrene. A 2-fold increase in v-fes integration resulted from benzo[a]pyrene pretreatment and pyrene posttreatment, while posttreatment with benzo[a]pyrene inhibited v-fes integration by about 2-fold. In some cases pre- and posttreatment with hydrazine relative to ST-FeSV infection and pretreatment with mono-methyl
hydrazine appeared to inhibit v-fes integration by 2-fold but this was not consistent.

Tsuruo and Baluda (1977) found that the copy number of RSV genome equivalents integrated per chicken-embryonic-fibroblast cell treated with 4-NQO at various time intervals before or after infection did not differ from the copy number in untreated but infected cells. In a similar fashion, exposure of secondary rat embryo fibroblasts to 7,12-DMBA or benzo[a]pyrene prior to infection with adenovirus also did not alter the extent or pattern of viral DNA integration (Dorsch-Häsler, 1980). However, because integration of the oncogenic DNA viruses is not highly ordered as it is in the retroviruses, comparisons of the effects of chemicals on DNA and RNA virus integration may not be valid. Accepted carcinogens did not consistently enhance or inhibit v-fes integration when added at 2 hrs before or 2 hrs after ST-FeSV infection.

Damage to cellular DNA by chemicals was proposed as a means for enhancement of viral transformation by creating an increased number of sites for integration (Hirai et al., 1974; Milo et al., 1978). This was also believed to be the cause for an apparent 2-fold enhancement of SV40 DNA per nucleus in Chinese hamster embryo cells treated with 4-NQO (Hirai et al., 1974). Pretreatment of D-550 cells with benzo[a]pyrene may have caused DNA-damage sites in which ST-FeSV proviral or v-fes DNA could integrate. However, the hydrazines and PANA did not enhance v-fes integration relative to untreated, infected-cells when administered either before or after infection with ST-FeSV.
Interaction of chemical carcinogens with cellular or proviral DNA may interfere with the precise order of proviral integration. Benzo[a]pyrene and, in some cases, hydrazine, were the only chemicals which inhibited v-fes integration coordinate with inhibition of transformation when administered at 2 hrs after ST-FeSV infection. Benzo[a]pyrene is metabolized by the cytochrome P-450 enzyme system into mutagenic metabolites which include the anti and syn isomers of 7,8-dihydrobenzo[a]pyrene-7,8-diol-9,10-epoxide. These epoxides bind primarily to guanine residues in nucleic acids and the relative levels of these adducts vary with the cell system under study (Theall et al., 1981). Most of the class of aromatic amines of which phenyl-α-naphthylamine (PANA) is a member, also form adducts with DNA. N-acetoxy-2-acetylaminofluorene is one of the most studied carcinogens in this class. It reacts predominantly with the C-8 position of guanine and causes considerable local destabilization and unwinding of the DNA (Chang et al., 1974; Fuchs and Duane, 1974). In contrast to benzo[a]pyrene, PANA was not inhibitory to v-fes integration in human cells.

The hydrazines usually had no effect on v-fes integration when administered at 2 hrs after ST-FeSV even though they all caused significant inhibition of virus transformation at non-toxic levels (Blakeslee, 1981; Blakeslee et al., 1983a; 1983c). Hydrazine, monomethyl hydrazine, and SDMH caused enhancement of virus transformation when D-550 cells were exposed 2 hrs before infection with ST-FeSV (Blakeslee, 1981). Enhancement of transformation was believed to be related to cell growth stimulation shown by these chemicals in dose
survival studies. Enhancement of v-fes integration was not observed when D-550 cells were pretreated with hydrazine or its derivatives at 2 hrs before infection with ST-FeSV (Table 2). Pre- or posttreatment with hydrazine and mono-methyl hydrazine pretreatment resulted in inconsistent decreases in v-fes integration relative to ST-FeSV-infected cells only. The inconsistency of hydrazine treatment on v-fes integration may be related to the fact that it is a weak carcinogen (as classified by the International Agency for Research on Cancer, 1974). Johnson (1982) found that transformation by herpes simplex type 2 virus was enhanced in mouse cells pretreated with hydrazine and SDMH. However, mono-methyl hydrazine and UDMH produced no enhancement of HSV-2 transformation with pretreatment.

The variety of effects of the hydrazines on virus transformation may be related to their divergent carcinogenic potency and modes of action within the cell. DNA-damaging potency varied over an approximate 30-fold range among sixteen different hydrazine derivatives including hydrazine, SDMH, and UDMH (Parodi et al., 1981). Carcinogenic potency varied over an approximate 1900-fold range, and was not correlated with mutagenic potency. Parodi et al. (1981) found that hydrazine, SDMH, and UDMH were among those hydrazines which induced significant DNA fragmentation in liver and lung of mice treated in vivo. The presence of $O^6$-methylguanine ($O^6$MG) and 7-methylguanine (7-MG) was correlated with hydrazine treatment. Hydrazine appears to alkylate nucleic acids indirectly through an active metabolite (Quintero-Ruiz et al., 1981). Fiala (1977) suggested that a common metabolite of carcinogenic methyl- or ethyl-substituted hydrazo, azo,
and azoxy compounds results in the formation of an ultimate alkylating carcinogen. Hydrazine has mutagenic potential (Kimball, 1977) which could alter cellular DNA and interfere with ST-FeSV integration or modify proviral DNA either before or after integration. Hydrazine was reported to cleave both thymine and cytosine rings, leading to a loss of pyrimidines, to reduce the 5,6 double bond of pyrimidines at pH 8.0 in the presence of \( \text{O}_2 \) and \( \text{Cu}^{+2} \) as catalysts (Brown et al., 1966), and to promote the conversion of cytosine to 4-hydrazine-2-pyrimidine (Lingens and Schneider-Bernlohr, 1965; 1966). Barrows et al. (1983) observed that 5-methylcytosine synthesis followed DNA synthesis during hydrazine toxicity, and formation of \( \text{7-MG} \) and \( \text{0}_6\text{MG} \) in liver DNA of hydrazine-treated rats. UDMH apparently does not bind to nucleic acids and mono-methyl hydrazine may do so only weakly (Godoy et al., 1983). Shank (1979) has reported the presence of N-7 alkyl guanine derivatives and undetectable amounts of \( \text{0}_6\text{MG} \) in DNA from livers of mono-methyl hydrazine-treated animals. Mono-methyl hydrazine and UDMH caused covalent binding to proteins, but little was known about the chemical entities responsible for both the nonenzymatic and enzymatic covalent binding to proteins (Godoy et al., 1983). Hydrazines react with proteins to split peptide groups, transforming the carbonyl component to the corresponding hydrazides, which could explain the nonenzymatic covalent binding to proteins (Juchau and Horita, 1972).

Since the hydrazines (other than hydrazine itself, in some cases) did not appear to alter v-fes integration when added after ST-FeSV infection (Table 2), inhibition of transformation might have
been caused by protein interactions or mutagenesis of proviral DNA. The highly efficient transformation of fibroblasts and epithelial cells in vitro by FeSV involves transformation-specific proteins which appear to be unique for each individual strain of FeSV (Barbacid et al., 1980b; Chen et al., 1981; Chen et al., 1983; Ruscetti et al., 1980; Van de Ven et al., 1980a).

The transformation-specific protein of ST-FeSV is a fusion protein consisting of a portion of the FeLV core gene polyprotein (gag) linked to the protein encoded by fes, the onc gene of GA- and ST-FeSV (Coffin et al., 1981; Sherr et al., 1978; Stephenson et al., 1977). Functional analysis of the ST-FeSV gag-fes gene product led to the identification of a protein kinase activity with specificity for tyrosine residues (Barbacid et al., 1980a; Reynolds et al., 1980; Van de Ven et al., 1980b). The gag-fes polyprotein of ST-FeSV appears to be functionally similar to the tyrosine kinases produced by transforming genes of several other retroviruses (Land et al., 1983). Cellular transformation by ST-FeSV causes an increased level of total cellular phosphotyrosine. This presumably leads to the phosphorylation of critical substrates necessary for initiating and maintaining the transformed phenotype (Barbacid et al., 1980a; Barbacid et al., 1981b; Reynolds et al., 1981a). Chemical carcinogens may interfere with the phosphorylation of critical viral or cellular substrates necessary for induction of the transformed state.

The 85,000 d (P85) transforming polyprotein of ST-FeSV possesses a single tyrosine acceptor site. This acceptor site, as well as two serine phosphorylation sites localized within the p12 (gag)
structural component of ST-FeSV P85, was phosphorylated in cells nonproductively transformed by ST-FeSV (Blomberg et al., 1981). It is not known whether phosphorylation of the tyrosine acceptor site in P85 is of functional significance in the transformation process. Other primary acceptor sites of cellular origin may also be involved. Carcinogens could possibly bind to these acceptor sites which would result in conformational changes preventing phosphorylation.

Reynolds et al. (1981) confirmed there is reduced epidermal growth factor (EGF) binding by ST-FeSV-transformed mink cells. The significance of the close correlation between EGF binding and tyrosine-specific protein kinase activity is not known. Such a correlation could reflect the existence of tyrosine acceptor sites within or close to the cellular receptor for EGF, phosphorylation of which would result in an impairment to EGF binding. It is also possible that cellular factors such as sarcoma growth factor (SGF) (Todaro and DeLarco, 1978) could be activated by phosphorylation of specific cellular tyrosine acceptor sites and compete with EGF binding surface receptors. Chemical carcinogens may cause membrane alterations or disturb receptor sites which are targets for phosphorylation or growth factors and impair transformation.

The protein kinase activities of several retroviruses were grouped into various classes based on cation and phosphate donor specificity (Snyder, 1982). The protein kinase of ST-FeSV prefers Mn$^{+2}$, but has a rather strict specificity for ATP as a phosphate donor and for adenosine nucleotides as effectors. It is not known whether the distinctions between the classes of protein kinases are
significant in an overall hypothesis about protein kinase-mediated transformation or whether these differences merely reflect the properties of the enzymes existing in the host cells from which the viruses originated. They could represent classes of protein kinases with very different primary substrate specificities in vivo. Chemical carcinogens might alter the primary substrate(s) for ST-FeSV protein kinase and cause it to phosphorylate alternate substrates.

The v-fes gene of ST-FeSV is homologous to v-fps, the transforming gene common to the Fujinami, PR C II, and UR I strains of avian sarcoma virus (Groffen et al., 1983b; Shibuya, 1980). The products encoded by v-fes and v-fps are immunologically and biochemically related (Barbacid et al., 1981a; Beemon, 1981) and all v-fes coding regions have a counterpart within the v-fps gene. The protein kinases encoded by v-fes and v-fps probably have equivalent locations and functions within the cell. The P130 protein encoded by gag-fps of Fujinami sarcoma virus is located at some areas of the plasma membrane, but is widely distributed in the cytoplasm. This suggests that the primary targets of the ST-FeSV and Fujinami sarcoma virus protein kinases are also widely distributed in the cytoplasm. P130 does not appear to be an integral membrane protein, but rather appears to interact with structural protein matrix (Feldman et al., 1983). Feldman et al. (1983) suggested that interaction with a structural protein matrix may be a general property of transforming proteins associated with tyrosine-specific protein kinase activities. Depending upon their time of treatment, chemicals may somehow
interfere in this interaction and circumvent or enhance the transformation process.

This study was primarily concerned with inhibition of transforming activity by ST-FeSV when carcinogens were added after virus infection. Certain recent studies reported that loss of the transformed phenotype was associated with increased cytosine methylation of proviral DNA. This is particularly interesting since 5-methyl-cytosine synthesis was reported as a consequence of hydrazine treatment (Barrows et al., 1983). Hydrazine and mono-methyl hydrazine both alkylate DNA indirectly, but only N-7 and O6MG were reported after treatment with mono-methyl hydrazine (Quintero-Ruiz et al., 1981; Shank, 1979).

Groffen et al (1983a) demonstrated a correlation between methylation of cytosine residues and expression of the major ST-FeSV proviral DNA translation product in transformed mink cells. Increased cytosine methylation was associated with loss of the transformed phenotype. The region subject to control by methylation was localized within the ST-FeSV proviral DNA itself. Expression of v-fes was not necessarily inhibited if integration occurred within a highly methylated region of the cellular genome. A similar correlation was observed between demethylation and expression of the transformed phenotype in mink cells transformed by v-abl (Abelson murine leukemia virus).

Similarly, McGeady et al. (1983) showed inhibition of the transforming activity of cloned Moloney MSV proviral DNA by in vitro methylation of cytosine residues. They concluded that methylation of
the v-mos gene was more inhibitory to transformation than methylation of the viral LTR, however, methylation of either of these sequences resulted in inhibition of transformation.

Another example of the effects of methylation of cytosine residues on retroviral expression was the lack of expression of Moloney MuLV in murine embryonal carcinoma (EC) cells. Although infected EC cells contained up to 100 integrated proviral genomes, the expression of these genes was suppressed. This inactivation was correlated with de novo methylation of the viral DNA, which may be a characteristic of early embryonic cells (Stewart et al., 1982).

Methylation of cytosine residues may regulate genes involved with expression of viral oncogene-mediated transformation and the expression of cellular homologues of viral oncogenes. Chemical carcinogens which alkylate cytosine directly were not used to treat human cells. However, post-treatment with hydrazine may have indirectly caused an increase in cytosine methylation of proviral DNA and subsequent inhibition of its expression. This would explain the fact that transformation was inhibited but v-fes integration was usually not.

In conclusion, the expectation that all chemical carcinogens interact in the ST-FeSV transformation process by a similar mechanism is highly unlikely. Cell growth and differentiation are generally controlled by a multicomponent system. Multiple membrane or protein alterations caused by chemicals and ST-FeSV infection could lead to a number of activities influencing cytoskeletal elements and possibly second messenger functions. Because all of the carcinogenic
chemicals (except benzo[a]pyrene and occasionally hydrazine) when added at 2 hrs after ST-FeSV infection caused inhibition of transformation without inhibiting v-fes integration (Table 2), they may have affected target sites or cellular substrates involved in the transformation process.

The procedures used to purify cellular DNA did not recover low mol. wgt. proviral DNA which had entered the nucleus but not integrated (Materials and Methods, p. 67-71). Panganiban and Temin (1983) recently reported that integration of retrovirus DNA is not a necessary prerequisite for retrovirus gene expression or replication. Inhibition of integration after entry into the nucleus seems highly unlikely in this study since all chemicals except benzo[a]pyrene (and occasionally hydrazine and mono-methyl hydrazine) demonstrated v-fes integration with an efficiency equal to that of ST-FeSV-infected cells only (Table 2). Also, none of the chemicals appeared to prevent synthesis and migration of proviral DNA into the nucleus. No unintegrated sequences with v-fes homology were detected within the cytoplasm of treated cells by 6 days after infection (Figures 14 and 15). Unintegrated proviral DNA might have been degraded by cytoplasmic nucleases by this time, however.

Cytoplasmic DNA was not screened for v-fes homology at times other than 6 days after infection. If chemicals were interfering with v-fes or proviral synthesis this might be more appropriately detected at less than 6 hrs after infection. Proviral DNA integration begins shortly after this time (Varmus et al., 1974; Gianni et
al., 1975) and interference with proviral synthesis becomes more
difficult to detect.

An attempt was made to determine whether v-fes integrated into
nonpermissive human fibroblasts as a unique sequence or as part of
altered or unaltered ST-FeSV proviral DNA. Few studies have investi-
gated the integration of retroviral DNA in nonpermissive, transformed
host cells. Varmus et al. (1973) assessed the integration of
RSV-specific DNA into the genomes of two types of nonpermissive,
mammalian cells transformed by RSV. At least one or more copies of
RSV proviral DNA appeared to be covalently integrated in the diploid
genomes of rat XC cells and mouse B77/3T3 cells. However, because
the viral genome was represented by as little as 30% in their
hybridization probe, they could not claim that the entire viral
genome was integrated as DNA. Tatosyan et al. (1983) analyzed four
different avian sarcoma virus (ASV)-transformed mammalian rat and
hamster cell types for the presence of ASV-specific sequences in
their genomic DNA. They concluded that the transformed phenotype of
the cells was controlled by src-specific sequences, but the structure
of the provirus itself underwent different changes. In some of the
provirus equivalents the src gene was intact, but in others it
appeared to be altered. Variable src-specific fragments were present
in both permissive and nonpermissive mammalian cells. There was no
correlation between the number of proviral copies and their level of
expression. In nonpermissive mammalian cells the ASV-proviral
sequences were found to be hypermethylated. If a similar situation
exists in the ST-FeSV - human fibroblast nonpermissive system,
provirus expression might similarly be affected by factors such as integrity of the provirus and its level of methylation. Chemical carcinogens might interpose to influence proviral rearrangements or methylation.

The v-fes sequence is cleaved from ST-FeSV proviral DNA by Pst I as two fragments of approximately 500 base pairs each, which together comprise about 80% of the v-fes gene (Franchini et al., 1981). It was anticipated that Pst I digestion of D-550 DNA infected with ST-FeSV and/or treated with chemicals would result in a 500 base pair fragment with homology to v-fes in Southern blot hybridizations. This fragment size did not show increases in homology to v-fes in addition to c-fes homology in normal background DNA (Results, p. 101). This indicated that v-fes might have rearranged after proviral integration in nonpermissive human cells. An alternative possibility was that the human DNA had become partially single-stranded during alkaline hydrolysis to remove RNA. This would have allowed cleavage by Pst I, but the restriction would not necessarily occur at the same locations within single-stranded DNA as in double-stranded DNA. Hence, it could not be determined with certainty whether v-fes DNA rearranged when nonpermissive human cells were infected with ST-FeSV in vitro. This would be of interest, for if such rearrangements occur, they might affect v-fes expression in human cells. Chemical carcinogens could further contribute to alterations in proviral DNA structure and function.
Figure 18.

Genomic maps of ST-FeLV and ST-FeSV proviral DNA oriented from 5' to 3' with respect to viral RNA. The double-stranded full-length linear DNA proviruses of ST-FeLV and ST-FeSV are approximately 8.5 and 5.0 kilobase pairs (Kbp), respectively. The extended terminal repeats (0.75 Kbp in length) are indicated by rectangles at the ends of the molecules. The heavy lines indicate common gene sequences shared by ST-FeLV and ST-FeSV including gag (group-specific antigens), the 5' portion of ST-FeLV env (envelope) gene, and the c region common to normal feline DNA. The pol gene coding for reverse transcriptase and the 3' end of the env gene are unique to ST-FeLV. The Fsrc or v-fes gene is unique to ST-FeSV and is shown as an internal rectangle. (Courtesy of Dr. C.J. Sherr, St. Jude Children's Research Hospital, Memphis, TN; Sherr et al., 1980.)
Proviral DNA

FeLV-ST

FeSV-ST

Figure 18
Figure 19.

Heteroduplex map of ST-FeLV and ST-FeSV in a 5' to 3' orientation with respect to viral RNA. The numbers above various segments indicate the average contour lengths in Kbp. The heavy lines indicate homology between shared gag, env, c, and extended terminal repeat sequences. The looped out regions are the unique pol (3.9) and env (1.1) gene sequences of ST-FeLV and the v-fes gene (1.5) of ST-FeSV. (Courtesy of Dr. C. J. Sherr, St. Jude Children's Research Hospital, Memphis, TN; Sherr et al., 1980).
FeLV: FeSV Heteroduplex

65.12% of FeSV homologous to FeLV
35.90% of FeLV homologous to FeSV
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