MOLECULAR INTERACTIONS OF HIGH ENERGY FUELS AND JET FUELS
WITH ONCOGENIC VIRUSES AND ENDOGENOUS VIRUSES

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The views and conclusions contained in this document are those of the authors and should not be interpreted as necessarily representing the official policies or endorsements, either expressed or implied, of the AFOSR of the U.S. Government.
The objectives of this research were to develop rapid in-vitro assays, to evaluate the carcinogenic potential of chemicals used by the U.S. Air Force. Snyder-Theilen Feline Sarcoma Virus (ST-FeSV), quantitatively transforms human skin fibroblasts following second order kinetics. These studies were performed in order to determine whether chemicals altered ST-FeSV transformation in a predictable manner and to correlate the alteration with the carcinogenic or non-carcinogenic activity of the test chemical.
Block 20—Abstract (continued)

The results, to date, show diverse carcinogens classed as: aromatic amines, polycyclic hydrocarbons, aminofluorenes, hydrazines, asbestos and mycotoxins inhibited virus transformation when virus infected cells (2 hours post-infection) were exposed to test chemical, while non-carcinogenic chemicals had no significant effect on transformation. Triton X-100, acetone, petroleum and shale oil derived JP5; RJ5 and diesel fuel, marine, demonstrated non-carcinogenic activity while formalin demonstrated carcinogenic activity. Experiments designed to show the specificity of the antagonistic effect of known carcinogens are reported. Disulfuram inhibits biotransformation of 1,2 symmetrical dimethyl hydrazine (SDMH) metabolites, azomethane to azoxymethane (ultimate carcinogen) thereby preventing carcinogenic effect of the proximate carcinogen SDMH. Cells treated with SDMH inhibited virus transformation whereas cells co-treated with SDM and disulfuram resulted in no significant difference in transformation frequency when compared to controls. Furthermore, the data show disulfuram failed to abrogate the inhibitory effect of MAMA, the ultimate carcinogen of SDMH.

Detailed methodology required to ascertain effect of chemicals on ST-FeSV provirus integration and synthesis were performed. The data show chemicals which inhibited ST-FeSV transformation reduced the number of copies of proviral DNA integrated into human cells. The inhibition of transformation thus appears to affect virus integration. This data correlated with a double-blind study in which inhibition of transformation detected 80% of chemical carcinogens tested.

A second in vitro assay is described. Isolation and purification of an endogenous feline virus, RD114 structural proteins designated P28, is described. P28 is expressed in all feline tissues and cells. Data show increased P28 expression resulted following in vitro exposure of feline embryo cells with 11 of 13 known carcinogens. Four non-carcinogens and two carcinogens did not significantly affect virus expression. Thus, 85% of the carcinogens significantly increased endogenous virus expression.

The data suggest both in vitro assay systems would effectively detect different classes of carcinogens with 80-85% accuracy.
Final Report

to

The Air Force Office of Scientific Research
Directorate of Life Sciences
Bolling Air Force Base
Washington, DC 20332

Title: Molecular Interactions of High Energy Fuels and Jet Fuels
with Oncogenic Viruses and Endogenous Viruses

Inclusive Dates of Report
July 1, 1980 to September 30, 1983

Submitted by:

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I. RESEARCH OBJECTIVES

The objectives of this research were to develop rapid short-term in vitro assays in order to evaluate carcinogenic and/or toxicologic chemicals used in world-wide U.S.A.F. operations. This type of predictive assay would greatly reduce the time and money required to determine these effects in in vivo systems.

Through the auspices of this contract, two systems were developed to determine carcinogenic potential. One system involved a human diploid cell transformation system with an oncogenic virus of feline origin and the second system involved the effect of diverse chemicals on endogenous virus gene expression. The first system was validated by a double-blind study in conjunction with the Chemical Research Resources Program, CRCB, DCCP, NCI, Bethesda, MD. The second system was not evaluated in a double-blind study, although, to date, chemical carcinogens increased expression of the endogenous feline virus, RD114 P28 core protein within 72 hours after treatment of feline embryo cells with known carcinogens at non-toxic levels.

II. SPECIFIC OBJECTIVES

The specific aims of this contract were to:

1. Develop and validate an in vitro transformation assay in human cells. (Years 1-3.)
2. Develop an in vitro transformation assay in feline cells. (Years 2-3.)
3. Evaluate molecular mechanisms of chemical-virus interactions by hybridization analysis of chemically-treated virus-infected human cells. (Years 1-3.)

All objectives were met and are contained in the following final report.
III. STATUS OF RESEARCH

Objective 1

The cell line used for this aspect of this program was Detroit 550, a human diploid skin fibroblast line from the American Type Culture Collection. The virus used was the Snyder-Theilen strain of feline sarcoma virus. This system was used to screen carcinogenic and non-carcinogenic chemicals. The major findings were that carcinogens inhibited virus transformation whereas non-carcinogens did not. Furthermore, the effect was not a result of cell killing in that non-toxic concentrations of chemicals were used for cell treatment. It was further shown that the carcinogens inhibited a specific virus gene function or product (v-fes), whereas other gene products or gene functions (gag, pol and FOCMA-S) were unaffected by chemical treatment as determined with the assay system used. The results showed carcinogens inhibited v-fes expression when virus-infected cells were exposed to the test chemicals 2 hours post-infection (p.i.) while non-carcinogens had no significant effect on virus transformation when added 2 hours p.i. (progress reports, years 1 and 2). In order to validate this system, a double-blind study was undertaken with Dr. David Longfellow, Chemical Resources Branch of the National Cancer Institute. The results are presented herein.

Materials and Methods

1. Cells. Detroit 550 human skin fibroblast (HSF) cells (normal male) (ATCC, CCL109, Rockville, Md) were grown in Minimal Essential Medium with Earle's salts, supplemented with 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 2 mM glutamine, 50 μg/ml Gentamicin and 10% fetal bovine serum (CM10) (with 5% serum, CM5; with no serum, CM0). Cultures were incubated at 37°C in a 5% CO₂ humidified atmosphere and routinely split at 7-day intervals. Cells used in transformation assays were between passage levels 15 through 30.
2. Virus. Snyder-Thilen feline sarcoma virus (ST-FeSV), initially obtained from tumor suspensions, was grown in feline embryo tissue culture. Clarified supernatants were stored in 0.5 ml aliquots at -85°C.

3. Chemicals. Cells were treated with chemicals at 2 hours or 6 hours post-virus-infection. Two non-toxic concentrations of each chemical (the low concentration a ten-fold dilution of the high) were used. Chemicals were dissolved in suitable solvents and diluted to required concentration in CM10. Twelve wells were used for each concentration and for each treatment time. Twenty-four wells were used for untreated, virus-infected controls. Cells were exposed to the chemicals for one hour at 37°C and were then washed in CM5 and refed with CM10 (1.5 ml/well). All chemical preparations and treatments were carried out under red light.

Solvents used were water, acetone, alcohol and DMSO. Solvent controls were run in those cases where alcohol or DMSO were used as they significantly affected the assay.

4. Toxicity assays. Cells were seeded into 35mm diameter wells at 150 cells/well in 4 ml CM10. Under red light, ten-fold dilutions of the chemical were made in CM10. Six wells were used for each dilution and twelve wells for untreated controls. Cells were exposed to the chemical for one hour at 37°C, then washed with CM5 and refed with CM10. After 4-6 days incubation, cells were fixed with 10% buffered formalin and stained with Giemsa. Clones containing at least 10 cells were counted.

5. Transformation assays. Confluent HSF cells were trypsinized and seeded into 16mm diameter wells at 3.5 x 10⁴ cells/well in 1.5 ml CM10 and incubated for 20 hours. The medium was aspirated and cells were treated with DEAE-Dextran (40 μg/ml in CM0) for 20 minutes at room temperature. The cells were then washed with CM5 and inoculated with 20-25 FFU ST-FeSV in 0.1 ml CM5/well. Plates were rocked at 20-minute intervals throughout the 2-hour, 37°C incubation period. The inoculum was aspirated and cells were refed with CM10. After 7-9 days incubation, cells were washed once with CM0, fixed with 10% buffered formalin
and stained with Giemsa. Foci of transformed cells were counted using a
dissecting microscope at 40X magnification. Data were evaluated by
Student's "t" test.

Results shown in Table 1A are 25 known chemical carinogens. Of
these, 20 significantly inhibited ST-FeSV transformation of HSF when
administered 2 hours post-infection (Fig. 1). Five carcinogens were
scored as negative in the assay and are shown in Table 2 as false neg-
atives. Shown in Table 1B are 13 non-carcinogenic chemicals of which 7
tested as negative in the assay. Six significantly inhibited transform-
ation under the test conditions. These are shown in Table 2 as false
positives. Solvents used in the assay are shown in Table 1C. DMSO
significantly inhibited transformation when administered 2 hours p.i.
Carcinogen/non-carcinogen pairs were also assayed (Table 3). A high
degree of correlation was seen with all the pairs tested with the excep-
tion of the carcinogen D,L-ethionine which registered as a false (-).

Conclusions and Significance

Twenty of 25 chemical carinogens and 6 of 13 non-carcinogens
significantly inhibited virus transformation of normal human skin fibro-
blasts when administered 2 hours after virus infection. Three of the 5
carcinogens scored as false negatives, namely Saffrole, benzidine and
4-aminazobenzene (Butter Yellow), require cellular activation systems
such as liver cells to biotransform the proximate carcinogen to the
ultimate carcinogen. It is possible the ultimate carcinogen was not
activated in this cell system. D,L-ethionine is recognized as a car-
inogen in rats, but not in hamsters. Therefore, its classification as
a carcinogen is questionable. CPCD also has been proven difficult to
detect in other in vitro assay systems.

Saffrole, Butter Yellow and CCPD all were dissolved in DMSO which,
by itself, reacts significantly as positive in this assay. Protein
kinases which regulate phosphorylation are known to control both normal
and neoplastic cell growth. Abnormal phosphorylation has been associ-
ated with transformation induced by retroviral protein kinases.
Recently, Rubin and Earp showed that DMSO stimulated phosphorylation of
170,000 d membrane protein. Others have shown DMSO to increase uptake of calcium ions, to cause changes in affinity for insulin receptors and to disrupt microfilaments— all factors in growth regulation.

Thus, the use of DMSO as a solvent in studies such as these may mask the results of chemical activity. For example, Benzo(e)pyrene dissolved in DMSO was negative, whereas when it was dissolved in acetone, it was scored as a positive, indicating carcinogenic activity. Benzo(j)pyrene was the only chemical which tested as positive when dissolved in DMSO.

Anthracene and pyrene are widely accepted as non-carcinogens. The expert committee which provided chemicals for the double-blind study reported that, although many in vitro studies on both anthracene and pyrene have been reported, the results have been inconclusive, thereby questioning the non-carcinogenic activity reported for these chemicals. Diphenyl nitroasmine has shown carcinogenic activity in several in vitro assays. Several studies showed retinol acetate (vitamin A) in high doses was teratogenic when fed to pregnant mice, whereas sodium ascorbate (vitamin C) and azoxybenzene may represent true false positives in that no evidence of carcinogeneity has been described for these chemicals.

The inhibition of ST-FeSV transformation by chemical carcinogens may reflect reduced levels of v-fes synthesis or v-fes integration. Alternatively, the target site of the v-fes gene product may be affected.
Figure 1

FINAL TEST PROTOCOL

NSF (P16-30)
3.5 x 10^4/well
16 hours

24 wells x n

20-30 FFU/well
ads. 120°

Chemical
60°
rinse
feed

incubate 7-9 days
fix, stain
count foci
<table>
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<tr>
<th>Chemical</th>
<th>Concentration</th>
<th>Solvents used</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. Carcinogens:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. α-Naphthylamine</td>
<td>10, 0.01 μg/ml</td>
<td>acetone</td>
</tr>
<tr>
<td>2. β-Naphthylamine</td>
<td>20, μg/ml</td>
<td>acetone</td>
</tr>
<tr>
<td>3. Phenyl(a)naphthylamine</td>
<td>10, 0.1 μg/ml</td>
<td>acetone</td>
</tr>
<tr>
<td>4. Phenyl(β)naphthylamine</td>
<td>10, 0.1 μg/ml</td>
<td>acetone</td>
</tr>
<tr>
<td>5. Hydrazine</td>
<td>60, 6 ppm</td>
<td>aqueous</td>
</tr>
<tr>
<td>6. Mono-methylhydrazine</td>
<td>100, 10 ppm</td>
<td>aqueous</td>
</tr>
<tr>
<td>7. Unsymmetrical dimethyl hydrazine</td>
<td>100, 10 ppm</td>
<td>aqueous</td>
</tr>
<tr>
<td>8. Symmetrical dimethyl hydrazine</td>
<td>200, 10 ppm</td>
<td>aqueous</td>
</tr>
<tr>
<td>9. N-Acetoxy 2 fluorenyl acetamide</td>
<td>100, 10 ppm</td>
<td>acetone</td>
</tr>
<tr>
<td>10. Benzo(a)pyrene</td>
<td>10, 1.0 μg/ml</td>
<td>acetone</td>
</tr>
<tr>
<td>11. Benzo(e)pyrene</td>
<td>10, 1.0 μg/ml</td>
<td>acetone &amp; DMSO</td>
</tr>
<tr>
<td>12. 4-Amino-biphenyl</td>
<td>10, 1.0 μg/ml</td>
<td>acetone</td>
</tr>
<tr>
<td>13. 4 dimethyl aminobenzene</td>
<td>10, 1 μg/ml</td>
<td>DMSO</td>
</tr>
<tr>
<td>14. N-Nitrosomethyl urea</td>
<td>10, 1 μg/ml</td>
<td>aqueous</td>
</tr>
<tr>
<td>15. Safrole</td>
<td>10, 1 μg/ml</td>
<td>DMSO</td>
</tr>
<tr>
<td>16. β-Propiolactone</td>
<td>10, 1 μg/ml</td>
<td>aqueous</td>
</tr>
<tr>
<td>17. 4,4′-methylene bis (2-chloroaniline)</td>
<td>10, 1 μg/ml</td>
<td>acetone</td>
</tr>
<tr>
<td>18. Benz(a)anthracene</td>
<td>10, 1 μg/ml</td>
<td>acetone</td>
</tr>
<tr>
<td>19. Benz(j)fluoranthene</td>
<td>10, 1 μg/ml</td>
<td>DMSO</td>
</tr>
<tr>
<td>20. Benzidine</td>
<td>10, 1 μg/ml</td>
<td>alcohol</td>
</tr>
<tr>
<td>21. D,L ethionine</td>
<td>10, 1 μg/ml</td>
<td>aqueous</td>
</tr>
<tr>
<td>22. Methyl azoxy methanol acetate</td>
<td>10, 1 μg/ml</td>
<td>acetone</td>
</tr>
<tr>
<td>23. Cyclopenteno(C,D)pyrene</td>
<td>10, 1 μg/ml</td>
<td>DMSO</td>
</tr>
<tr>
<td>24. Formaldehyde</td>
<td>10, 1 ppm</td>
<td>aqueous</td>
</tr>
<tr>
<td>25. N-(2-fluorenyl) acetamide</td>
<td>10, 1 μg/ml</td>
<td>acetone</td>
</tr>
<tr>
<td><strong>B. Non-Carcinogens:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. N-(4-fluorenyl) acetamide</td>
<td>10, 1 μg/ml</td>
<td>acetone</td>
</tr>
<tr>
<td>2. Pyrene</td>
<td>10, 1 μg/ml</td>
<td>acetone</td>
</tr>
<tr>
<td>3. 2-Aminobiphenyl</td>
<td>10, 1 μg/ml</td>
<td>acetone</td>
</tr>
<tr>
<td>4. Anthracene</td>
<td>10, 1 μg/ml</td>
<td>acetone</td>
</tr>
<tr>
<td>5. Phenanthrene*</td>
<td>10, 1 μg/ml</td>
<td>acetone</td>
</tr>
<tr>
<td>6. Retinol acetate (Vit. A)*</td>
<td>10, 1 μg/ml</td>
<td>alcohol</td>
</tr>
<tr>
<td>7. Sodium ascorbate*</td>
<td>10, 1 μg/ml</td>
<td>aqueous</td>
</tr>
<tr>
<td>8. L-Methionine*</td>
<td>10, 1 μg/ml</td>
<td>aqueous</td>
</tr>
<tr>
<td>9. Azoxybenzene*</td>
<td>10, 1 μg/ml</td>
<td>alcohol</td>
</tr>
<tr>
<td>10. Diphenyl nitrosamine*</td>
<td>10, 1 μg/ml</td>
<td>acetone</td>
</tr>
<tr>
<td>11. Gamma butyrolactone*</td>
<td>10, 1 μg/ml</td>
<td>aqueous</td>
</tr>
<tr>
<td>12. Acetone</td>
<td>2x10⁴, 2x10³, 1x10³, 1x10² ppm</td>
<td>aqueous</td>
</tr>
<tr>
<td>13. Ethyl alcohol</td>
<td>1x10³, 1x10² ppm</td>
<td>aqueous</td>
</tr>
<tr>
<td>14. DMSO</td>
<td>1x10³, 1x10² ppm</td>
<td>aqueous</td>
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TABLE 2. FALSE NEGATIVES AND FALSE POSITIVES DETECTED IN ASSAY

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<tr>
<th>Chemicals</th>
<th>False Positive</th>
<th>False Negative</th>
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<tr>
<td>Safrole*</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Benzidine</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>D,L Ethionine</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Cyclopenteno(C,D)Pyrene*</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>4-Dimethyl Aminobenzene</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Retinol Acetate</td>
<td>+</td>
<td>~</td>
</tr>
<tr>
<td>Sodium Ascorbate</td>
<td>+</td>
<td>~</td>
</tr>
<tr>
<td>Azoxy Benzene</td>
<td>+</td>
<td>~</td>
</tr>
<tr>
<td>Diphenyl Nitrosamine</td>
<td>+</td>
<td>~</td>
</tr>
<tr>
<td>Anthracene</td>
<td>+</td>
<td>~</td>
</tr>
<tr>
<td>Pyrene</td>
<td>+</td>
<td>~</td>
</tr>
</tbody>
</table>

* Dissolved in DSMO
### TABLE 3. TEST OF CARCINOGEN/NON-CARCINOGEN PAIRS

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>In Vivo Activity</th>
<th>FeSV Test Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 2-aminobiphenyl</td>
<td>NC</td>
<td>-</td>
</tr>
<tr>
<td>4-aminobiphenyl</td>
<td>C</td>
<td>+</td>
</tr>
<tr>
<td>2. γ-butyrolactone*</td>
<td>NC</td>
<td>-</td>
</tr>
<tr>
<td>β-propiolactone*</td>
<td>C</td>
<td>+</td>
</tr>
<tr>
<td>3. N-4 fluorenylacetamide*</td>
<td>NC</td>
<td>-</td>
</tr>
<tr>
<td>N-acetoxy 2-fluorenylacetamide*</td>
<td>C</td>
<td>+</td>
</tr>
<tr>
<td>4. Pyrene*</td>
<td>NC</td>
<td>-</td>
</tr>
<tr>
<td>Benzo(a)pyrene*</td>
<td>C</td>
<td>+</td>
</tr>
<tr>
<td>5. L-methionine*</td>
<td>NC</td>
<td>-</td>
</tr>
<tr>
<td>D,L-ethionine*</td>
<td>C</td>
<td>False(-)</td>
</tr>
</tbody>
</table>

* Double blind
Objective 2

A second in vitro assay system developed to assay carcinogens involved the detection of a specific viral core protein, p28, in feline embryo cells. This core protein of an endogenous xenotropic feline virus, RD114, is found in all normal feline cells. P28 levels were detected with a competitive radioimmunoassay using donkey anti-p28 antisera and 125I-p28 following exposure of feline embryo cells for 24, 48 and 72 hours to chemical carcinogens and non-carcinogens. The results of this study are reported herein.

Materials and Methods

Isolation of RD114/p28 Structural Protein. The human cell line, rhabdosarcoma (RD) was grown in RPMI 1640 medium (Gibco), supplemented with fetal bovine serum (Hyclone). The cells were seeded, then fed at 48-hour intervals with RPMI 1640, supplemented at the first and second feeding with 10% FBS and with 5% FBS thereafter. At 20 days, the cells were harvested and the harvested culture medium was then clarified by centrifugation at 1000 rpm for 10 minutes. The medium was concentrated to a volume of 200 ml using a Millipore Pellicon Cassette System and then ultracentrifuged at 100,000 g for 90 minutes to pellet the virus. The virus was resuspended and purified by centrifugation (100,000 g, 60 minutes) in a 20-50% continuous sucrose gradient. The virus was then freeze-thawed twice, followed by ultracentrifugation at 100,000 g for 90 minutes. The pellet was then resuspended in a TKE-D-TX buffer (0.05M Tris HCl, 0.05M Tris base, 0.6M KC1, 0.01 M EDTA, 0.01M Dithriothreitol, 1% Triton X-100) and incubated at 37°C for 60 minutes. The resuspended pellet was centrifuged again (100,000 g, 90 minutes). The supernatant was poured off, extracted with ether, bubbled with nitrogen, and then ultracentrifuged at 100,000 g for 90 minutes. The resulting supernatant was chromatographed on Biogel P-100 (BioRad) with an eluting buffer of 0.5M phosphate buffer pH 7.5 (column K1.5 x 30, sample size 1.5 ml, fraction size 1 ml). The RD114/p28 protein elutes in the second peak (Fig. 1.). Each fraction in the second eluted peak was tested for the
presence of p28 by SDS gel electrophoresis. Those fractions shown to contain only p28 were pooled and stored at -80°C. Concentration of p28 was determined by Lowry Protein Assay using BSA as a standard.

**Lowry Protein Assay.** A solution (hereafter termed Regent A) was made up containing 0.5 ml of 1% cupric sulfate and 0.5 ml of 2% potassium tartrate per 50 ml of 2% sodium carbonate. Standard tubes were made up containing 20, 40, 80, 120, 160 and 200 mg, respectively, of bovine serum albumin in 5 ml of Regent A. 0.5 ml of the solution to be tested was also added to 5 ml of Regent A and all tubes were allowed to stand at room temperature for 10 minutes. 0.5 ml of 1N phenol reagent was then added to each tube and after 30 minutes the color which developed was read in a spectrophotometer at a wavelength of 750 nm.

**Iodination.** Ten μg purified p28 and 100 μg of Chloramine-T were added to a combi vial containing 1 mCi of Na-125. The contents of the vial were mixed for 60 seconds and the reaction was stopped by the addition of 250 μg of sodium metabisulfite. The iodination reaction was then quenched by the addition of 1 mg KI and 0.1 mg bovine serum albumin. The mixture was then chromatographed on a Biogel DG-50 column (0.9 x 30 cm) to separate 125I-p28 from free iodine. The eluting buffer was composed of 0.01M Tris HCl pH 7.8, 0.4M NaCl, 0.3% Triton X-100, 0.1% sodium azide and 1% bovine serum albumin. One ml fractions were collected and counted. Fractions from the protein peak (the first peak eluted) were pooled and diluted with elution buffer to a final concentration of 100 ng/ml, then stored at 4°C.

**Treatment of FE Cells with Carcinogen.** The FE cells were seeded into T75 flasks (Corning) with McCoy's 5A medium and 10% fetal bovine serum (FBS). The cells were then incubated at 37°C for 24 hours after passage. The culture medium was then removed and replaced with 5 ml of medium containing the carcinogen to be tested at a concentration determined by prior toxicity studies. The cells were then incubated with the carcinogen for 30 minutes. At the end of 30 minutes the carcinogen-containing media was removed, the cells were rinsed with 10 ml of
McCoy's and incubated again in 30 ml McCoy's supplemented with 10% FBS. One non-treated control flask and 1 flask at each chemical concentration were harvested at 27-hour intervals (24, 48 and 72 hours after treat-
ment). Harvesting was accomplished as follows: the cells were rinsed twice with 10 ml McCoy's, then treated with 3 ml of 0.25% trypsin. Detached cells were then suspended in 5 ml McCoy's with 10% FBS and pelleted by centrifugation at 1000 rpm for 10 minutes. The supernatant was then poured off and cells were resuspended in 5 ml buffer (0.01M Tris HC1 pH 7.8, 0.1M NaCl, 0.05M EDTA, 0.5% Triton X-100) and kept on ice. The cells were then sonicated for 30 seconds and ultracentrifuged at 35,000 rpm for 60 minutes. The resulting supernatant is stored at -80°C and tested for the presence of the p28 protein by radioimmunoassay and for total protein concentration by the Lowry modification of the Folin-Ciocalteau protein assay.

**Competitive Radioimmunoassay.** The competitive radioimmunoassay is performed by the double antibody method. Reagents used are the following:

- **Antigen:** p28 at 20 µg/ml; 125I-p28 at 1 ng/ml, specific activity 25 µCi/µg buffer: 0.01M Tris HC1 pH 7.8, 0.4M NaCl, 0.3% Triton X-100, 0.1% sodium azide, 1% bovine serum albumin.

- **Primary antibody:** goat anti-p28 antiserum buffer: 0.01M Tris HC1 pH 7.8, 0.03M EDTA, 0.4M NaCl, 0.3% Triton X-100, 0.1% sodium azide, 1% bovine serum albumin, 1% normal goat serum.

- **Secondary antibody:** donkey anti-goat antiserum buffer: 0.01M Tris HC1 pH 7.8, 0.001M EDTA, 0.1M NaCl, 0.1% Triton X-100.

Serial two-fold dilutions of the cold antigen were made giving final concentrations ranging from 4.88 ng/ml to 625 ng/ml. 0.05 ml aliquots of each dilution were added to 0.05 ml of antiserum which has been diluted 1/500. 0.05 ml samples of each solution to be tested for p28 levels were also added to 0.05 ml of antisera. Control tubes were also defined as follows: a total count tube contained 0.05 ml of antigen buffer and 0.05 ml of primary antibody buffer, a background count tube contained 0.05 ml of antigen buffer and 0.05 ml of primary
antibody buffer, and a reference count tube contained 0.05 ml of antigen buffer and 0.05 ml of primary antibody. All tubes were then incubated at 4°C. Twenty-four hours later 0.1 ml of the hot antigen, 125I-p28, was added to all tubes. The tubes were again incubated at 4°C for 24 hours. On day 3, 0.2 ml of undiluted secondary antibody was added to all tubes except the total count control tubes and all tubes were again incubated at 4°C for 24 hours. At that time the tubes were "harvested" by the following procedure: all tubes were diluted out to 1 ml by the addition of 0.8 ml of secondary antibody buffer. The tubes were then centrifuged at 2500 rpm for 15 minutes, the supernatant was aspirated off of all tubes except the total count control tubes and the tubes were counted in a gamma counter (Micromedic, counting time 1 minute). A standard curve was created by plotting antigen concentration against counts bound/counts in reference count control tube and unknown p28 levels were calculated from the standard curve required to determined confidence levels.

Results

The first experiment initiated was the determination of passage effects on p28 expression in feline embryo cells. As shown in Table 1, p28 expression was monitored through 16 passages. P28 protein concentrations ranged from 0.257 at passage 1 to 1.47 at passage 16 (ng/mg total cellular protein) with a mean value of 0.993 ng/mg and a standard deviation of 0.390. FE cells were treated with different chemicals and p28 levels were determined at 3 time periods. The chemicals used, concentrations and graphical representations are shown in Table 2.
TABLE 1.  RD114 P28 EXPRESSION IN NORMAL FELINE CELLS
FROM PASSAGE 1 THROUGH 16.

<table>
<thead>
<tr>
<th>Passage Level</th>
<th>Concentration ng/mg total protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.257</td>
</tr>
<tr>
<td>2</td>
<td>N.D.</td>
</tr>
<tr>
<td>3</td>
<td>N.D.</td>
</tr>
<tr>
<td>4</td>
<td>0.422</td>
</tr>
<tr>
<td>5</td>
<td>N.D.</td>
</tr>
<tr>
<td>6</td>
<td>N.D.</td>
</tr>
<tr>
<td>7</td>
<td>1.04</td>
</tr>
<tr>
<td>8</td>
<td>1.03</td>
</tr>
<tr>
<td>9</td>
<td>1.39</td>
</tr>
<tr>
<td>10</td>
<td>1.18</td>
</tr>
<tr>
<td>11</td>
<td>N.D.</td>
</tr>
<tr>
<td>12</td>
<td>1.11</td>
</tr>
<tr>
<td>13</td>
<td>1.20</td>
</tr>
<tr>
<td>14</td>
<td>N.D.</td>
</tr>
<tr>
<td>15</td>
<td>1.47 Mean=0.933</td>
</tr>
<tr>
<td>16</td>
<td>0.838 S.D.=0.390</td>
</tr>
</tbody>
</table>

1. Passage level - confluent monolayers trypsinized and split at 1:3 ratios.
2. N.D. = Not Determined.
TABLE 2. CHEMICALS AND DOSE LEVELS USED IN FE CELLS

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Concentration</th>
<th>Figure No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrazine</td>
<td>5 &amp; 50 ppm</td>
<td>2</td>
</tr>
<tr>
<td>SDMH</td>
<td>10 &amp; 100 µg/ml</td>
<td>3</td>
</tr>
<tr>
<td>UDMH</td>
<td>10 &amp; 100 ppm</td>
<td>4</td>
</tr>
<tr>
<td>MMH</td>
<td>10 &amp; 100 ppm</td>
<td>5</td>
</tr>
<tr>
<td>N94-Fluorenyl) acetamide</td>
<td>10 &amp; 1 µg/ml</td>
<td>6</td>
</tr>
<tr>
<td>Pyrene</td>
<td>15 &amp; 5 µg/ml</td>
<td>7</td>
</tr>
<tr>
<td>BAP</td>
<td>15 &amp; 5 µg/ml</td>
<td>8</td>
</tr>
<tr>
<td>Beta-naphthylamine</td>
<td>15 &amp; 5 µg/ml</td>
<td>9</td>
</tr>
<tr>
<td>Ph.-beta naphthylamine</td>
<td>15 &amp; 5 µg/ml</td>
<td>10</td>
</tr>
<tr>
<td>Ph.-alpha naphthylamine</td>
<td>15 &amp; 5 µg/ml</td>
<td>11</td>
</tr>
<tr>
<td>N-acetoxy AAF</td>
<td>1. &amp; .1 µg/ml</td>
<td>12</td>
</tr>
<tr>
<td>Acetone</td>
<td>2 &amp; .2%</td>
<td>13</td>
</tr>
<tr>
<td>Formalin</td>
<td>10 &amp; 1 ppm</td>
<td>14</td>
</tr>
<tr>
<td>MNNG</td>
<td>0.1 &amp; 0.01 µg/ml</td>
<td>15</td>
</tr>
<tr>
<td>4 NQO</td>
<td>0.001 &amp; .0001 µg/ml</td>
<td>16</td>
</tr>
<tr>
<td>4 ABP</td>
<td>10 &amp; 1 µg/ml</td>
<td>17</td>
</tr>
<tr>
<td>2 ABP</td>
<td>10 &amp; 1 µg/ml</td>
<td>18</td>
</tr>
</tbody>
</table>

The figures 2-18 are graphical representations showing the effect of the above listed chemicals on p28 expression.

Results

Shown in Table 3, FE cells treated with 11 carcinogens increased p28 expression. Enhanced expression varied by values ranging from a 2.1-fold increase with 0.0001 µg/ml 4NQO to a 35-fold increase with 100 µg/ml of SDMH. Maximum expression occurred after 48 or 72 hours incubation. These results are shown graphically in Figures 2-18. No effect was noted after 24 hours incubation with any of the test chemicals. Furthermore, 4 non-carcinogens had little or no effect on expression in addition to 2 carcinogens. Thus, 11 of 13 known carcinogens enhanced expression of an endogenous viral protein whereas 3 non-carcinogens did not. Thus, 85% of the carcinogens were detectable with this assay system. There is some question as to the carcinogenic potential of formalin and, if this data were included in the non-carcinogen class, the percent of detectable carcinogens would be increased to 92% levels. Further studies are required to determine confidence levels.
<table>
<thead>
<tr>
<th>Fig.</th>
<th>Maximum Increase ng/ml</th>
<th>Time (hrs)</th>
<th>Concentration</th>
<th>Exp.</th>
<th>Control</th>
<th>Ratio Exp./Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Hydrazine (C)&lt;sup&gt;1&lt;/sup&gt;</td>
<td>1</td>
<td>48</td>
<td>5 ppm</td>
<td>932.5</td>
<td>31.2</td>
</tr>
<tr>
<td>2.</td>
<td>SDMH (C)</td>
<td>2</td>
<td>48</td>
<td>100 µg</td>
<td>1065.75</td>
<td>30.41</td>
</tr>
<tr>
<td>3.</td>
<td>UDMH (C)</td>
<td>3</td>
<td>72</td>
<td>100 ppm</td>
<td>1526.1</td>
<td>56.6</td>
</tr>
<tr>
<td>4.</td>
<td>MMH (C)</td>
<td>4</td>
<td>48</td>
<td>100 ppm</td>
<td>579.8</td>
<td>196.3</td>
</tr>
<tr>
<td>5.</td>
<td>BAP</td>
<td>7</td>
<td>72</td>
<td>15 µg</td>
<td>2426.5</td>
<td>974.4</td>
</tr>
<tr>
<td>6.</td>
<td>β-Naphthylamine (C)</td>
<td>8</td>
<td>72</td>
<td>15 µg</td>
<td>1037.0</td>
<td>370.7</td>
</tr>
<tr>
<td>7.</td>
<td>Phenyl-β-naphthylamine (C)</td>
<td>9</td>
<td>72</td>
<td>15 µg</td>
<td>1153.3</td>
<td>246.8</td>
</tr>
<tr>
<td>8.</td>
<td>N-AC-AAF (C)</td>
<td>11</td>
<td>72</td>
<td>0.1 µg</td>
<td>633.9</td>
<td>29.6</td>
</tr>
<tr>
<td>9.</td>
<td>MNNG (C)</td>
<td>14</td>
<td>72</td>
<td>0.01 µg</td>
<td>206.2</td>
<td>68.7</td>
</tr>
<tr>
<td>10.</td>
<td>4 APB (C)</td>
<td>16</td>
<td>72</td>
<td>10 µg</td>
<td>165.7</td>
<td>55.01</td>
</tr>
<tr>
<td>11.</td>
<td>4 NQO (C)</td>
<td>15</td>
<td>72</td>
<td>0.0001 µg</td>
<td>168.3</td>
<td>81.6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fig.</th>
<th>Maximum Increase ng/ml</th>
<th>Time (hrs)</th>
<th>Concentration</th>
<th>Exp.</th>
<th>Control</th>
<th>Ratio Exp./Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>N-4 fluorenyl acetamide (NC)&lt;sup&gt;2&lt;/sup&gt;</td>
<td>5</td>
<td>72</td>
<td>1 µg</td>
<td>282.5</td>
<td>207.6</td>
</tr>
<tr>
<td>2.</td>
<td>Pyrene (NC)</td>
<td>6</td>
<td>72</td>
<td>5 µg</td>
<td>583.3</td>
<td>567.6</td>
</tr>
<tr>
<td>3.</td>
<td>Acetone (NC)</td>
<td>12</td>
<td>72</td>
<td>2%</td>
<td>45.2</td>
<td>49.4</td>
</tr>
<tr>
<td>4.</td>
<td>Formalin (C?)</td>
<td>13</td>
<td>72</td>
<td>1 ppm</td>
<td>54.0</td>
<td>53.8</td>
</tr>
<tr>
<td>5.</td>
<td>2 ABP (NC)</td>
<td>17</td>
<td>72</td>
<td>10 µg</td>
<td>32.3</td>
<td>40.3</td>
</tr>
<tr>
<td>6.</td>
<td>Phenyl-α-naphthylamine (C)</td>
<td>10</td>
<td>72</td>
<td>15 µg</td>
<td>1541.7</td>
<td>894.2</td>
</tr>
</tbody>
</table>

<sup>1</sup> C = carcinogen.

<sup>2</sup> NC = non-carcinogen.
Figure 1. Chromatographic separation of iodinated RD-114 P28 fraction from free iodine.
OBJECTIVE 2

Figures 2-18

Legend: (■) represents "high" dose; (X--X) "low" and (--) control.
Figure 2

Incubation Time in Hours

Hydrazine (5 & 50 PPM)

MG P2B/MG Total Cellular Protein
INCUBATION TIME IN HOURS

SDMH (10 & 100 UG/ML)

NG P28/MG TOTAL CELLULAR PROTEIN

Figure 3
Figure 4
NG P28/MG TOTAL CELLULAR PROTEIN

HOURS AFTER TREATMENT
PYRENE (5 AND 15 UG/ML)

Figure 7
BETA NAPTHYLAMINE (5.815 UG/ML)

INCUBATION TIME IN HOURS

NG P28/MG TOTAL CELLULAR PROTEIN

Figure 9
NG P28/MG TOTAL CELLULAR PROTEIN

INCUBATION TIME IN HOURS
PH-BETA NAPTHYLAMINE (5 & 15 UG/ML)

Figure 10
NG P28/MG TOTAL CELLULAR PROTEIN

INCUBATION TIME IN HOURS

PH-ALPHA NAPHTHYLAMINE (5 & 15UG/ML)

Figure 11
Figure 14
Figure 16

NG P28/MG TOTAL CELLULAR PROTEIN

HOURS AFTER TREATMENT
4NQO (0.0001 AND 0.001 UG/ML)
Figure 17

NG P28/MG TOTAL CELLULAR PROTEIN

HOURS AFTER TREATMENT
4ABP (1 AND 10 UG/ML)
Objective 3

Evaluation of Molecular Mechanisms of Chemical-Virus Interactions by Hybridization Analysis of Chemical-Treated Virus-Infected Cells.

Introduction

This total research project has been concerned with the effects of chemical carcinogens (at various time periods and at non-toxic doses) on ST-FeSV initiated transformation of human skin fibroblasts (Detroit 55 - ATCC) in vitro. Known carcinogens, representative of various classes, which have been used in the cocarcinogenic test system developed in this laboratory have inhibited transformation of human fibroblasts following their exposure to the test chemical at two hours post-infection with ST-FeSV (see Table 1). Non-carcinogens have had no significant effect on transformation at two hours post-infection (9,10).

TABLE 1. CORRELATION BETWEEN INHIBITION OF VIRUS TRANSFORMATION AND DIVERSE CHEMICALS INDUCING TUMORS IN RODENTS

<table>
<thead>
<tr>
<th>Chemical Treatment</th>
<th>Reported Activity&lt;sup&gt;†&lt;/sup&gt;</th>
<th>Inhibition of ST-FeSV Transformation</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Aromatic amines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. Naphthyl amines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Two (2)</td>
<td>C</td>
<td>yes</td>
</tr>
<tr>
<td>2. Phenyl-alpha</td>
<td>C</td>
<td>yes</td>
</tr>
<tr>
<td>3. Phenyl-beta</td>
<td>C</td>
<td>yes</td>
</tr>
<tr>
<td>4. N-Acetoxy 2fluoranylacetamide</td>
<td>C</td>
<td>yes</td>
</tr>
<tr>
<td>II. Polycyclic Hydrocarbons</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Benzo[a]pyrene</td>
<td>C</td>
<td>yes</td>
</tr>
<tr>
<td>2. Pyrene</td>
<td>NC</td>
<td>no</td>
</tr>
<tr>
<td>III. Hydrazines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Hydrazine</td>
<td>C</td>
<td>yes</td>
</tr>
<tr>
<td>2. Mono-methyl hydrazine</td>
<td>C</td>
<td>yes</td>
</tr>
<tr>
<td>3. 1,1 Dimethyl hydrazine</td>
<td>C</td>
<td>yes</td>
</tr>
<tr>
<td>4. 1,2 Dimethyl hydrazine</td>
<td>C</td>
<td>yes</td>
</tr>
<tr>
<td>IV. Other</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Aflatoxin B&lt;sub&gt;1&lt;/sub&gt;</td>
<td>C</td>
<td>yes</td>
</tr>
<tr>
<td>2. Amosite asbestos</td>
<td>C</td>
<td>yes</td>
</tr>
<tr>
<td>3. JPS (shale)</td>
<td>NC</td>
<td>no</td>
</tr>
<tr>
<td>4. JPS (petrol)</td>
<td>NC</td>
<td>no</td>
</tr>
<tr>
<td>5. RJS</td>
<td>NC</td>
<td>no</td>
</tr>
<tr>
<td>6. Diesel fuel, marine</td>
<td>NC</td>
<td>no</td>
</tr>
<tr>
<td>7. Acetone</td>
<td>NC</td>
<td>no</td>
</tr>
<tr>
<td>8. Methyl azoxymethanol-acetate</td>
<td>C</td>
<td>yes</td>
</tr>
<tr>
<td>9. Formalin</td>
<td>C</td>
<td>yes</td>
</tr>
<tr>
<td>10. Triton X-100</td>
<td>?</td>
<td>no</td>
</tr>
</tbody>
</table>

<sup>†</sup>C, Carcinogen; NC, non-carcinogen.
Additional studies have indicated that known carcinogens may be inhibiting expression of the specific retroviral transforming gene known as v-fes in ST-FeSV. Other viral gene products (gag, pol, and FOCHA-S) have appeared to be unaltered in the presence of carcinogens (11).

Test results have suggested the possible value of this fibroblast-ST-FeSV system as a rapid, predictable in vitro assay for evaluation of chemicals in regard to carcinogenic potential. The results of a double-blind evaluation of this assay system conducted in conjunction with the Chemical Carcinogen Reference Standard Repository, Chemical and Physical Branch, DCCP, NCI are presented elsewhere in this report.

The specific aspects of the ST-FeSV chemical interaction which this section of the report deals with are whether or not carcinogenic chemicals may interfere with proviral synthesis or integration into the host cell genome when administered at two hours post-infection with ST-FeSV. This is the critical period at which transformation is inhibited. It is known that by two hours after infection, retroviruses, in general, begin synthesizing complementary proviral DNA from their RNA in the host cell cytoplasm and initiate its transport and integration into the nucleus.

This study is of value in understanding chemical-viral interactions in several ways:

1) In providing insight into the mechanistic reasons for chemical alteration of the transforming capacity of ST-FeSV and possibly other retroviruses.

2) In providing supporting evidence for the usefulness of the fibroblast-ST-FeSV test system, developed in this laboratory, as a predictable indicator of carcinogenic chemicals in a screening program.

3) In providing a broader basis of understanding about the effects of carcinogens on proviral DNA (retroviruses) or oncogenic DNA (adenoviruses, herpesviruses, and polyoma viruses) synthesis and integration as variables of time of infection with these respective viruses.

4) In providing clues to the effects of carcinogenic chemicals in the environment on in vivo expression of both vertically-transmitted endogenous retroviruses and horizontally-transmitted exogenous retroviruses or oncogenic DNA viruses.
This study relates to the current interest in viral-chemical interaction sometimes referred to as syncarcinogenesis or cocarcinogenesis in the oncogenic process. Chemicals and known carcinogens have been observed to enhance or negate virus expression or the oncogenic potential of transforming viruses depending upon such variables as: 1) the in vitro or in vivo system employed, 2) the transforming virus used for infection (RNA or DNA virus) or the particular endogenous virus present in the system, 3) the time of chemical administration relative to virus infection, 4) the concentration of chemical used, and 5) the mode of action of the particular carcinogen or tumor promoter used for treatment.

It is known that the frequency of transformation by DNA tumor viruses can be enhanced by exposing cells to irradiation, thymidine analogs, and chemical carcinogens of various classes. The molecular basis by which these agents enhance transformation is not known (65). In the adenovirus (Simian adenovirus 7)-hamster embryo cell system, either enhanced or inhibited transformation frequencies were observed relative to the concentration and time of administration of chemicals (16). This is similar to our findings in the ST-FeSV-human fibroblast system. Both physical and chemical carcinogens have been shown to mediate the amplification of SV40 DNA synthesis in the Chinese hamster embryo cell system. However, not all of the viral DNA sequences were amplified. Only certain sequences of the viral genome were present in amplified amounts and were present in DNA molecules which contained both SV40 and hamster DNA sequences. This may reflect a general gene amplification phenomenon mediated by carcinogens (62, 63, 64). Similarly, tumor-promoting substances were shown to amplify Epstein-Barr virus early antigen (EA) expression by non-virus producer Raji cells. This system has been proposed as a short-term in vitro assay for tumor promoters (54).
Treatment of cells with chemical carcinogens and mutagens has resulted in different rescuability of integrated endogenously and exogenously acquired type C retroviruses. In most cases, this has been determined not to be due to differences in virus penetration (among a given cell population) or to enhanced susceptibility to infection by a chemically resistant cell population. Derepression of certain oncogenic retroviruses may depend on the dose of chemical used. It has been suggested that induction of endogenous viruses could lead to expression of silent genes or the formation of recombinant transforming viruses. Cell cycle stages also have been shown to significantly affect chemical and hormonal induction of endogenous murine retroviruses (85).

Very little work has been done specifically with quantitating genome integration by transforming viruses in host systems treated with chemical carcinogens. It was found that treatment of Chinese hamster embryo cells with the carcinogen 4 nitroquinoline 1-oxide (4-NQO) two hours prior to infection with SV40, resulted in: 1) increased cell transformation frequency, 2) increased levels of nuclear penetration by SV40, and 3) two-fold enhancement of the levels of SV40 DNA integrated into cellular DNA as compared to controls (53). 4-NQO is known to produce DNA strand scission which may facilitate integration. In addition, 4-NQO might render nonpermissive cells permissive for viral replication, increasing the number of molecules of SV40 DNA available for integration, or 4-NQO may modify 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 hydrocarbon (PAH) carcinogens prior to infection with adenovirus 5 (also a DNA-transforming virus), showed enhanced viral transformation but no alteration in the extent of viral DNA synthesis or integration (29).

Another study, with which our research problem may have more similarities, was also conducted with a retrovirus, specifically Rous sarcoma virus. In this case, the copy number or viral genome equivalents integrated per cell (chicken embryonic fibroblast) treated with UV radiation or 4-NQO at various time intervals before or after infection

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did not differ from that in untreated, infected cells. From this study, it appears that the formation and repair of random single strand breaks and gaps in cellular DNA did not facilitate integration of proviral DNA. It was concluded in this case that integration appears to be restricted to specific sites and suggests that the reaction is probably carried out by a specific enzymatic mechanism (91).

Some of the chemicals used in our human fibroblast-ST-FeSV system may have modes of action in addition to those discussed above in various other studies. The purpose of this section of the research is to determine whether a difference exists in the number of genome copies of ST-FeSV (specifically v-fes) integrated into host cells in the presence of chemicals added either previous to or post-viral infection relative to untreated, ST-FeSV infected human fibroblast cells. If carcinogenic chemicals do not affect integration in our system, proviral synthesis, transport of proviral DNA into the nucleus, or repression of integrated proviral DNA may be affected, leading to inhibition of transformation by any one or more of these mechanisms.

The Initial Method of Approach Used to Detect Integrated ST-FeSV in Human Fibroblasts.

These methods, used previous to January 1983, involved the following steps:

1) The culture and purification of feline leukemia virus Kawakami-Theilen strain (KT-FeLV).
2) Purification of viral RNA from KT-FeLV.
3) Preparation of a radiolabeled complementary DNA (C-DNA) copy of purified KT-FeLV RNA in an exogenous in vitro reaction using avian myeloblastosis virus reverse transcriptase.
4) The use of the single stranded C-DNA probe in liquid hybridization studies to detect homologous sequences in human fibroblast DNA purified from cells previously infected with ST-FeSV and treated with chemicals at critical time periods.

See diagram of this methodology on page 42.
This approach was discontinued previous to the liquid hybridization studies due to multiple problems arising from the use of KT-FeLV as a probe for the \textit{v-fes} function of ST-FeSV and due to critical problems with denaturation of KT-FeLV RNA and its unsuitability as a C-DNA template. The methodology and results from the above approach will be given, followed by a substantiating list of reasons for rejecting this approach in favor of a more direct set of methods which could better answer the same questions.

A) Culture and Purification of KT-FeLV Culture:

1) FL-74 cells which continually produce and shed KT-FeLV were grown in suspension culture in 0.15 gallon disposable glass roller bottles. The complete medium used consisted of the following:

- RPMI-1640 (GIBCO) containing 0.2% sodium bicarbonate plus -15% Newborn Calf Serum (Sterile Systems)
- 1% L-Glutamine
- 2,000 units Penicillin/ml
- 0.1% Gentocin

The roller bottles were incubated at 37°C on a roller apparatus. Cells were seeded weekly (7-day intervals) at 1.2 to 1.4 x 10^6 cells/ml in 200 ml of the RPMI-1640 complete medium per roller bottle. On day 5 the cells were fed an additional 200 ml of the RPMI-1640 complete medium per roller bottle.

The medium, containing shed virus, was harvested on day 8 and cells were removed by centrifugation at 1500 RPM for 15 minutes prior to virus purification.

2) The preceding culture regime was later modified. FL-74 cells were seeded at 2.5 x 10^6 cells/ml in seven liters of complete RPMI-1640 medium. The cells were constantly suspended with slow stirring in a 15 liter glass Bellco spinner flask at 37°C. The culture medium containing virus was harvested after only twelve hours of growth and immediately clarified by means of centrifugation.
FeLV-KT

FeLV-RNA

5' 70S 5'

Exogenous Reaction

AMV Reverse Transcriptase

32P-C DNA

Human D_{550} Cell DNA (ds)

Infected with FeSV (FeLV)-ST and Treated with Chemicals

Proviral DNA

Sonicate Denature Hybridize

Analyze by Reassociation Kinetics

42
FeLV-KT Production

FL-74 Cells

RPMI - 1640: 15% Newborn CS
1% L-Glutamine
2,000 μ/ml Penicillin
0.1% Gentocin

Roller Bottles

Seeded 1.2 - 1.4 x 10^6 cells/ml
Feed on day 5
Harvest day 7

Bellco Spinner Flask

Seeded 2.5 x 10^6 cells/ml
Harvest at 12 hours.

Purification (Based on References 13, 88, and 89):

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

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

3) The viral pellets were resuspended in 1X TNE buffer, pH 7.4 (0.01 M Tris, 0.1 M NaCl, and 0.001 M EDTA) at 0°C. (From the original 15 liters, 12 pellets were obtained and resuspended in 5 ml of 1X TNE per pellet.)
4) The resuspended viral pellets were then layered onto 20-50% linear sucrose density gradients prepared in 1X TNE, pH 7.4 and centrifuged at 25,000 RPM for 1 hour at 4°C in the SW 27 rotor. (Five ml virus suspension is layered onto each of twelve 30 ml gradients).

5) The dense viral bands which sediment approximately half way down in the gradients were harvested manually from above with a long needle and syringe against a background of reflected light.

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 were sufficient to reduce the sucrose concentration to less than 1%).

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

8) The virus pellets were suspended in a total of 15 ml of 1X TNE, pH 7.4 and were stored frozen at -80°C prior to extraction of the RNA.

See the following flow chart of the purification procedure.

FeLV-KT Purification

1. Culture medium
   2. Discard Cells → 600 x g
   3. Millipore-Pellicon Filter Unit
      concentrate 10-15 fold
   4. Centrifugation (25,000 RPM
      SW 27 Rotor
      1 hour)
   5. Supernatant → Virus Pellets
      Resuspended in 1X TNE
   6. 20-50% Sucrose Density Gradients
   7. Dialysis against 1X TNE
   8. Ultracentrifugation

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B) Extraction and purification Procedure for KT-FeLV RNA

(Based on References 13, 79, and 88.)

Methods:

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

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

2) An equal volume of Proteinase K (Boehringer Mannheim; self-digested at 60°C for 20 minutes) at 1000 μg/ml in 1X TNE pH 7.4 was added such that the final proteinase K concentration was 500 μg/ml. This mixture was incubated for 30 minutes 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 minutes to 1 hour.

4) The mixture was centrifuged at 1800 RPM for 10 minutes to separate the upper aqueous phase and lower, more dense phenol fraction. 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 0.2 M with Na acetate by addition of one-tenth volume of 2 M stock Na acetate (nuclease-free).

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

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

9) The RNA pellets were carefully decanted, drained and resuspended in as small a volume of 1X TNE, pH 7.4, as possible (approximately 200 to 300 μl per pellet).

10) The RNA was then layered onto 15-30% linear sucrose (nuclease-free) gradients prepared in 1X TNE, pH 7.4. Approximately 300 μl were layered onto 11.2 ml gradients.

11) Gradients were fractionated and scanned with an ISCO density gradient fractionator and UV monitor.
12) The peak fractions representing the viral 35 S RNA were collected separately and the RNA was reprecipitated by adding one-tenth volume of 2.0 M Na acetate and two volumes of absolute ethanol, and storing at 20°C.

13) The precipitated 70 S viral RNA was collected as in step 8 and subjected to a second cycle in sucrose gradients (steps 8-12 are repeated a second time). The final 70 S viral RNA preparation was isolated following centrifugation, resuspended in 1X TNE, pH 8.3, and used to prepare a complementary DNA product.

Following is a flow chart of the KT-FeLV RNA isolation and purification procedure.

**FeLV-KT RNA Isolation**

FeLV-KT in 1X TNE

1% SDS
500 µg/ml Proteinase K

37°C for 30 minutes

TNE saturated Phenol

3 X

Aqueous Phase

2 volumes Absolute EtOH
0.2 M Na Acetate

6,000 RPM, SS34
90 minutes

15-30% Sucrose Density Gradients
0.2% SDS

38,000 RPM, SW41
3.75 hours.

35S and 70S RNA
Analysis of the RNA purified from KT-FeLV

Background:

Retroviruses, in general, possess a 60-70 S single-stranded RNA genome which exists as a dimer of two genetically identical RNA subunits or molecules (28-35 S) (97). The functional significance of the dimer is unknown and both subunits may function in replication. A region of H-bonding holds the two subunits together. This region of H-bonding occurs near the 5' end of each subunit molecule but its precise location and structure are not absolutely known.

Other smaller molecules of lesser importance are thought to be associated with retrovirus RNA through H-bonding, forming a type of aggregate. These include a specific t-RNA associated with each dimer subunit and which binds to the viral RNA dependent DNA polymerase and functions in replication (35, 37). The RNA aggregate may also involve cellular 4 S t-RNAs which are carried along with the virus as it buds from the cell, as well as various R-RNAs (5 S, 18 S, and 28 S) which are cellular in origin (15, 19, 31, 36, 55, 61, and 67).

Brian et al. (13) have analyzed the RNA of the Rickard strain of FeLV produced by a permanently infected feline thymus tumor line (F-422). They identified three size classes of RNA by gel electrophoresis. These included: 1) a $6.2 \times 10^6$ to $7.1 \times 10^6$ mol. wgt. class sedimenting at 50-60 S and comprising 57% to 76% of the total viral RNA; 2) an $8.7 \times 10^4$ mol. wgt. class corresponding to the 8 S RNA reported in murine sarcoma virus RNA (35, 61); 3) a $2.5 \times 10^4$ mol. wgt. class sedimenting with 4 S cells: t-RNA and comprising 6% to 12% of the total viral RNA. If they assumed that the 50 to 60 S molecule exists at the rate of one per virion, then the 8 S molecule is present to the extent of 2 to 7 per virion, and the 4 to 5 S molecule is present to the extent of 20 to 60 per virion. Brian et al. (13) concluded that the 50 to 60 S ($6.2 \times 10^6$ to $7.1 \times 10^6$ mol. wgt.) RNA of FeLV-Rickard strain is composed 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 (58) have also analyzed the RNA and its subunits from various feline sarcoma-leukemia virus mixtures. They found that RNA from the Snyder-Theilen feline sarcoma-leukemia virus complex.
ST-FeSV-FeLV) sedimented in a double-peaked band from 50-70 S. Gardner-Arnstein (GA) FeSV-FeLV RNA sedimented as a single 70 S peak. ST-FeLV possessed RNA sedimenting as a single 70 S band, but F 422 FeLV RNA sedimented more slowly at 50 to 60 S. After using thermal denaturation to reveal subunit classes of RNA, they found that 3 subunit classes existed in ST-FeSV-FeLV RNA: 35-37 S, 32-34 S, and 25 S. ST-FeLV possessed the 35-37 S subunit. The second class of 32-34 S was similar to subunits found in F 422 FeLV RNA and minor components of GA-FeSV-FeLV and ST-FeLV RNA. The 25 S subunit class was detected only in ST-FeSV-FeLV RNA.

Figure 1

FeLV-KT RNA

E. coli Markers

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Because of the above findings, the evidence for KT-FeLV possessing an RNA genome composed of identical subunit or dimer structure was strong. Evidence for this was obtained by our sedimentation analysis of purified RNA from FeLV-KT on sucrose density gradients (see Figure 1) in which two peaks exist with sedimentation values of approximately 35 S and 70 S.

We chose to use the 35 S subunit rather than the 70 S molecule as a template in preparing a complementary DNA copy in vitro. In theory, the use of the subunit would provide additional RNA for use as a template and its shorter length (relative to the 70 S molecule) would allow for greater probability of continuous and complete copying by the reverse transcriptase enzyme.

**Figure 2**

![Graph showing FeLV-KT RNA sedimentation analysis](image)

![Graph showing heated 70S FeLV-RNA sedimentation analysis](image)
Methods generally used to dissociate the retrovirus RNA aggregate molecule into its separate subunits have included treatments with heat, urea, formamide, formaldehyde, and dimethyl sulfoxide (3, 23, 30, and 58). We used heating to dissociate the 70 S RNA of KT-FeLV into its component subunits. The 70 S RNA (twice purified through sucrose density gradients) in 0.01 N Tris, 0.1 N NaCl, 0.0001 M EDTA - 0.2% SDS, pH 7.4, was heated in boiling water for 45 seconds, chilled on ice, and analyzed in 15-30% sucrose density gradients (see Figure 2) as used for preparative isolation of the 70 S RNA previously.

Results:

The heated 70 S RNA sedimented in a rather broad band in the top one-third of the gradients with a sedimentation range of approximately 5-25 S (see Figure 2 on page 49). This indicated that the RNA no longer represented intact 35 S subunits but had dissociated into a range of many smaller pieces after heat denaturation. Further analyses using different heating times produced similar results in all cases. The 70 S RNA molecule had shown no evidence of nuclease degradation or nicking in preparative isolation procedures in sucrose gradients. It sedimented at the 70 S position as determined by the use of 16 S and 23 S markers from E. coli. However, a broad, highly absorbing (260 nm) peak was always present in the top one-third of the gradients (see Figure 1 on page 48) which provided some evidence that there was degradation of total RNA isolated from the FeLV preparations.

We became aware of the fact that the age of the FeLV at the time of purification was critical in obtaining RNA free of nicking due to nucleases (Paul Kimball - Battelle Memorial Institute, Columbus, Ohio - Personal Communication). Brian et al. (13) have noted variation in the characteristics of FeLV RNA (Rickard strain) with age of the virus. They found that the electrophoretic mobility of the 50 to 60 S molecule was variable depending upon the length of time the virus was grown. High molecular weight RNA from virus which had been labeled for 20 hours had an apparent mol. wgt. of $7.1 \times 10^6$ whereas RNA from virus labeled for 4 hours had an apparent mol. wgt. of $6.4 \times 10^6$. East et al. (33) had similar findings in which the sedimentation coefficients for the Rickard strain of FeLV were measured to be 58 S and 50 S, respectively, for 20-hour and 2-hour-old viral RNA.
Several workers have attempted to explain such discrepancies on the basis of a matruational process in which the RNA undergoes modification in the virion after budding, causing it to sediment at a faster rate (15, 19, 32, 33). Immature Rous sarcoma virus (3 to 60 minutes old) contains subunits from 15 to 60 S which apparently assemble into the larger 68 S molecule.

Assembly of a 58 S molecule in murine sarcoma virus (32) and FeLV (33) is suggested to occur from two or more 50 S molecules. Brian et al. (13) postulate that even though no RNA species ranging from 8 to 50 S were identified in FeLV harvested at 1 hour intervals or after 4 hours labeling, that any assembly process occurring between 4 and 20 hours which gives rise to a 60 S molecule would seem to occur between two or more 50 S species or a 50 S and smaller (4 to 8 S) molecule.

Brian et al. (13) also offered a further explanation. The denatured high molecular weight subunits from 20-hour FeLV RNA were shown to electrophorese and sediment with a more heterogenous pattern than the same RNA species from 4-hour-old viruses. They felt it was possible that nicks harbored within the loops of an aggregate molecule (20-hour) might allow secondary structural changes to occur (perhaps ends of loops would become more tightly folded) thereby creating a slower electrophoretically migrating or faster sedimenting molecule compared to the unnicked aggregate (4-hour). It has been shown that 60 to 70 S molecules of Rous sarcoma virus RNA apparently retain their aggregate structure in the presence of nicks which are revealed only after the aggregate has been denatured (2).

We felt this could explain the phenomenon which we observed with KT-FeLV RNA. Even though the 70 S molecule was nicked, such nicks were not revealed in sucrose density sedimentation analyses until the molecule was heat denatured. Due to the extreme age of the virus we used initially (7 days old) the RNA probably was highly assembled into an aggregate structure and held together in 70 S form through a high degree of intermolecular H-bonding, which was dissociated on heating. Therefore, such nicks were only revealed by denaturation studies as
evidenced by the broad band of low molecular weight RNA molecules which we observed in sucrose density gradient profiles. Apparently such RNA degradation occurs in FeLV grown for more than 3 hours (Paul Kimball, Battelle Memorial Institute, Columbus, Ohio; and Leland Velicer, Michigan State University, East Lansing, Michigan - Personal Communications). The KT-FeLV RNA which we had been isolating (even 12-hour cultures) was not suitable for preparing a complementary DNA copy because the nicked regions precluded continuous copying of the full length of the RNA molecule.

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 hours other than a lower recovery with 12-hour-old virus. Denaturation of the RNA is a risk with FeLV grown in culture for more than 3 hours. The ability to obtain sufficient quantities of FeLV using such short culture times essentially requires automatic harvesting equipment (Leland Velicer, Michigan State University, East Lansing, Michigan - Personal Communication).
**Preparation of radiolabeled complementary DNA (C-DNA) to KT-FeLV**

(Based on References 22, 72, 78.)

**Methods**

The following components were added in order to the final 1 ml reaction.

<table>
<thead>
<tr>
<th></th>
<th>Stock Concentration</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Tris-HCl, pH 8.1</td>
<td>500 mM</td>
</tr>
<tr>
<td></td>
<td>40 µl</td>
<td>50 mM</td>
</tr>
<tr>
<td>2</td>
<td>Double Distilled Deionized H$_2$O 120 µl</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>MgCl$_2$ 10 µl</td>
<td>800 mM in H$_2$O</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8 mM</td>
</tr>
<tr>
<td>4</td>
<td>dATP</td>
<td>10 mM in 50 mM</td>
</tr>
<tr>
<td></td>
<td>dTTP</td>
<td>Tris-HCl, pH 8.1</td>
</tr>
<tr>
<td></td>
<td>dGTP</td>
<td>1 mM each</td>
</tr>
<tr>
<td>5</td>
<td>$^{32}$P-dCTP</td>
<td>2-4 mCi</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(300-400 Ci/mM specific activity)</td>
</tr>
<tr>
<td>6</td>
<td>2-mercaptopethanol</td>
<td>2 M in H$_2$O</td>
</tr>
<tr>
<td></td>
<td>10 µl</td>
<td>20 mM</td>
</tr>
<tr>
<td>7</td>
<td>FeLV RNA, 35 S or 70 S 200 µl</td>
<td>250 µg/ml in 50 mM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tris-HCl, pH 8.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50 µg/ml</td>
</tr>
<tr>
<td>8</td>
<td>Calf Thymus Primers</td>
<td>2.5 mg/200 µl</td>
</tr>
<tr>
<td></td>
<td>200 µl</td>
<td>in 50 mM Tris-HCl, pH 8.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.5 mg/ml</td>
</tr>
<tr>
<td>9</td>
<td>Bentonite-80 µl</td>
<td>1 mg/ml in H$_2$O</td>
</tr>
<tr>
<td></td>
<td></td>
<td>80 µg/ml</td>
</tr>
<tr>
<td>10</td>
<td>AMV Reverse Transcriptase 40 µl</td>
<td>19,000 units/ml in Non-Tris buffer</td>
</tr>
<tr>
<td></td>
<td></td>
<td>750 units/ml</td>
</tr>
<tr>
<td>11</td>
<td>KCl-100 µl</td>
<td>1.4 M in H$_2$O</td>
</tr>
<tr>
<td></td>
<td></td>
<td>140 mM</td>
</tr>
</tbody>
</table>

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Incubation was for 3 hours at 37°C in capped plastic microfuge tubes which were siliconized and treated for nuclease contamination.

The oligonucleotides of the calf thymus DNA primer mixture were generated by digestion of 1.0 mg of calf thymus DNA (Sigma) with 70 μg DNase I (Sigma) per ml in a reaction containing 10 mM Tris-HCl, pH 7.4 and 10 mM MgCl₂ at 37°C for 2 hours. Afterward the DNase I was inactivated by heating at 121°C for 10 minutes. The initial DNA concentration was used as the measure of the primer concentration. (Active primers in such a mixture are approximately 8-15 nucleotides in length).

The following procedures which were used to terminate the C-DNA reaction. (These methods were based on those of Retzel et al., Ref. 72.)

1) Following the three-hour incubation, an equal volume of a solution consisting 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 minutes at 37°C.

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

3) The 5 ml mixture was extracted with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1). The phenol was first saturated with 50 mM Tris-HCl, pH 8.1. Extraction was for 30 minutes at room temperature with gentle stirring. The mixture was then centrifuged at 2,000 RPM for 15 minutes and the upper aqueous phase was collected.

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

5) The aqueous phase containing the C-DNA was placed at -20°C after adding 2 volumes of cold absolute ethanol and one-tenth volume of 2 M Na acetate to precipitate the C-DNA.

6) The single-stranded C-DNA was collected by centrifugation and separated from calf thymus primers and unincorporated nucleoside triphosphates by gel filtration on Sephadex G-50 using 50 mM Tris-HCl for elution at pH 8.1.
7) The pure C-DNA was precipitated again at -20°C with ethanol and Na acetate and was concentrated by centrifugation, resuspended in a small volume of 50 mM Tris-Cl, pH 8.1 and stored at -80°C.

**Analyses of Complementary DNA Products on Agarose Gels for Size Determination** (Based on the Methods of McMaster and Carmichael, Ref. 70.)

This method employed denaturation of nucleic acids and their reaction with glyoxal, followed by electrophoresis in a slab gel system. Glyoxalation introduces an additional ring onto guanosine residues which sterically hinders the formation of G-C base pairs and therefore the renaturation of native structure, allowing for a much more accurate determination of molecular weight.

DNA samples were incubated in capped plastic microfuge tubes (nuclease-treated and siliconized) for one hour at 50°C in 1.0 M glyoxal, 50% (vol/vol) dimethyl sulfoxide (Me₂SO), and 10 mM NaH₂PO₄/Na₂HPO₄, pH 7.0. Control samples were treated identically but in the absence of glyoxal and Me₂SO. 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 (Bio-Rad).

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 NaH₂PO₄/Na₂HPO₄, pH 7.0 which also served as the running buffer. Electrophoresis was at a constant current of 45 mAmps for two hours at room temperature. Bromophenol blue at 0.25% in 50% glycerol served as a marker dye. DNA molecular weight markers included the Eco R I and Hind III digests of Lambda DNA (Boehringer Mannheim).

We found that the C-DNAs obtained from both 35 S and 70 S RNAs were the same size regardless of whether the virus was grown for a short culture time (12 hours) or a much longer time (7 days). The glyoxal treatment used to denature the C-DNA for sizing in gels provided evidence that the C-DNAs existed as a heterogeneous, small size range with the
majority predominantly less than about 500 bases in size. Accurate size
determinations were difficult as no-good, single-stranded DNA markers
were available commercially and alkaline denaturation of double-stranded
DNA markers results in a smear in the gel due to nicking of these com-
mercial preparations during storage. The short length copies of C-DNA
which we obtained to KT-FeLV RNA were not very suitable as hybridization
probes and reflected the denatured state of the RNA used as a template.

Reasons for Abandoning the Use of C-DNA Prepared to KT-FeLV as a Probe
for ST-FeVL Proviral DNA Synthesized and/or Integrated into Human Skin
Fibroblasts.

Approximately only 35.90% of the ST-FeLV genome is homologous to
that of ST-FeSV RNA (79). The degree of homology between KT-FeLV and
ST-FeSV is less well known. Homologous regions of ST-FeLV and ST-FeSV
include the gag region of the 5' end which codes for group specific
antigens, a portion of the 5' end of the envelope gene, and the small c
or common region of the 3' end which represents a sequence also present
in normal cat cellular DNA. Please see the following Figures 3 and 4 on
pages 57 and 58 which demonstrate the homology between the 8.5 Kilobase
pair (Kbp) genome of ST-FeLV and the 5.0 Kbp genome of ST-FeSV (79).

The pol region which codes for reverse transcriptase function is
not present in FeSV and the unique v-fes or sarcoma gene function of
FeSV has no counterpart in the helper leukemia virus. It is known 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 tem-
plate and then jumps to the 3' LTR before proceeding with the synthesis
of the minus DNA proviral strand in a 3' to 5' direction (95).

The conditions of our complementary DNA synthesis in vitro allowed
only for a single-stranded copy to be made which was not full length due
to degradation of the viral RNA. Such a C-DNA to FeLV RNA would possess
very little homology to FeSV RNA including only a small region of the
gag gene, the c region, and possibly a small portion of the env gene.
Figure 3

Provirial DNA

FeLV-ST

ETR

gag | 2 | 3 | pol | 4 | 5 |

FeSV-ST

ETR

gag | F-src | 2 | 3 | env | 4 | ETR | v - fes

FeLV: FeSV Heteroduplex

Numbers on map are in terms of Kilobase pairs.

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

Furthermore, all of these regions would be present in the FeLV proviral DNA which integrated at the time of FeSV infection. FeSV always exists in nature as mixture with the helper FeLV which furnishes the pol and env functions which it lacks. Hence a C-DNA to FeLV RNA does not provide a specific probe for the transforming or v-fes function of ST-FeSV.

Due to the time restrictions causing our lack of capability to produce the large quantities of 3-hour-old FeLV required for an intact, undegraded RNA template, and due to the inherent problems in probing for integrated v-fes sequences with a C-DNA probe to FeLV-RNA, it was decided to adopt a new approach to this research problem.

Use of Cloned v-fes as a Probe in Identifying ST-FeSV Proviral DNA in Human Skin Fibroblasts by Dot-Blot Hybridization

The cloning of the v-fes gene of ST-FeSV provided a valuable tool in improving the specificity of FeSV proviral DNA detection in our research study. The cloned v-fes gene which we used was provided as a gift from Dr. Genoveffa Franchini at the National Cancer Institute. We were, in fact, provided with two separate clones prepared in pBR322 plasmid. The first of these clones, referred to as S_L, includes the left (5'-3') Pst I digestion fragment of the v-fes region and the second, referred to as S_R, includes the right Pst I digestion fragment of the v-fes region. Each fragment (approximately 0.5 Kilobase pairs long) was separately subcloned into the ampicillin-resistance gene of the plasmid vector pBR322 (39). The S_L cloned region was used throughout our studies to detect v-fes homology.

The methodology for this new approach involved basically four different steps:

1) Human skin fibroblasts (Detroit 550-ATCC) were grown in vitro and treated with known chemical carcinogens or noncarcinogenic analogs at two hours pre- or post-infection with ST-FeSV.
2) Nuclear and cytoplasmic fractions were isolated from chemically treated and ST-FeSV infected fibroblast and these were extracted and purified for subsequent sonication, sizing and quantitation of the DNA from each.

3) The v-fes gene cloned in plasmid pBR322 was propagated in E. coli K802 strain and was subsequently isolated and purified. Plasmid pBR322 (native, no cloned genes) provided by Dr. John Reeve, The Ohio State University, Columbus, Ohio, was propagated in E. coli D5410 strain. Both plasmid pBR322 and isolated v-fes DNA were radiolabelled with $^{32}$P by Nick-translation for use as probes in nucleic acid hybridization studies.

4) Dot-Blot hybridization (56, 87) of $^{32}$P-labelled plasmid or v-fes DNA to cellular DNA was done to detect homology or to quantitate the genome numbers of v-fes which were present in host nuclear or cytoplasmic DNA. Dot-Blot hybridization was chosen as an alternative to liquid hybridization to avoid problems with hybridization kinetics and for its convenience and economy in terms of the amounts of nucleic acid required.

A. Methodology

1) Culture and Cocarcinogensis Transformation of D550 Human Fibroblast Cells

a) Culture of Detroit 550 Human Fibroblast Cells
(American Type Culture Collection).

These cells were grown in Corning 490 cm$^2$ tissue culture roller flasks on a roller apparatus at 37°C in the following complete medium:

- Minimum Essential Medium-Earle's Salts (MEM-E, GIBCO)
- 0.22% Na Bicarbonate
- 10% Fetal Calf Serum
- 1% Non-Essential Amino Acids
- 1% Sodium Pyruvate
- 1% L-Glutamine
- 1% Gentocin

(100 ml per Flask.)
Cells were seeded at approximately $6 \times 10^6$ cells per roller flask and grown to confluency. The cells from each confluent flask were passaged into 2 to 3 flasks until adequate numbers were grown for cocarcinogenesis transformation studies.

b) Cocarcinogenesis Transformation of Detroit 550 Cells

Forty 490 cm$^2$ tissue culture roller flasks at approximately 80% confluency and between passages 20 to 30 ($10 \times 10^6$ cells per flask) were used for each transformation experiment to provide sufficient cellular DNA for subsequent hybridization experiments. Following is the list of compounds which were administered in different experiments, their concentrations, and time of administration relative to ST-FeSV infection.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Final Concentration</th>
<th>Time of Chemical Treatment Relative to Infection with 6.0 $\times$ 10$^3$ Focusing Forming Units of ST-FeSV (in 5 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrazine</td>
<td>60 pp</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 $\mu$g/ml</td>
<td>- 2 and + 2 hours</td>
</tr>
<tr>
<td>Unsymmetrical Dimethyl Hydrazine</td>
<td>100 $\mu$g/ml</td>
<td>- 2 and + 2 hours</td>
</tr>
<tr>
<td>Benzo(a)pyrene</td>
<td>10 $\mu$g/ml</td>
<td>- 2 and + 2 hours</td>
</tr>
<tr>
<td>Pyrene</td>
<td>10 $\mu$g/ml</td>
<td>- 2 and + 2 hours</td>
</tr>
<tr>
<td>Phenyl-$\alpha$-Naphthylamine</td>
<td>10 $\mu$g/ml</td>
<td>+ 2 hours</td>
</tr>
</tbody>
</table>

Detroit 550 cells—untreated with chemicals and uninfected, and Detroit 550 cell infected with ST-FeSV only and untreated will serve as controls.
The step-by-step procedures involved in the cocarcinogenesis of Detroit 550 cells were as follows: Forty roller flasks of D 550 cells at 80% confluency of growth (about $10 \times 10^6$ cells per flask) were used for each experiment.

Procedure for Administration of Compound 2 Hours Prior to ST-FeSV Infection.

1) Aspirate medium from roller flasks.
2) Add 20 ml of compound (cocarcinogen) at the desired concentration in complete MEM-E/Na medium containing 10% fetal bovine serum (FBS) to each roller flask and incubate 37°C for 1.5 hours.
3) Aspirate compound (cocarcinogen) off and wash with complete MEM-E/Na.
4) Add DEAE-Dextran at 40 µg/ml prepared in complete MEM-E/Na 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.) Incubate at 37°C for 20 minutes.
5) Aspirate off DEAE-Dextran.
6) Infect D 550 cells with ST-FeSV at approximately $1.22 \times 10^3$ focus forming units/ml (FFU/ml) in MEM-E/Na medium containing 5% FBS using 5 ml per culture flask. Incubate at 37°C for 2 hours.
7) Aspirate off virus.
8) Add 50 ml of complete MEM-E/Na medium (10% FBS) to each culture flask. Allow cells to grow an additional 6 days at 37°C.
9) Harvest cells by trypsinization, pellet by low speed centrifugation (1000 RPM for 10 minutes), and store frozen at -80°C.

Procedure for Administration of Compound 2 Hours After ST-FeSV Infection.

1) Aspirate medium from roller flasks.
2) Add DEAE-Dextran at 40 µg/ml prepared in complete MEM-E/Na medium (no FBS) using 5 ml per culture flask. Incubate at 37°C for 20 minutes.
3) Aspirate off DEAE-Dextran.
4) Infect with ST-FeSV at $1.22 \times 10^3$ FFU/ml in MEM-E/Na medium containing 5% FBS using 5 ml per culture flask. Incubate at 37°C for 2 hours.
5) Aspirate off virus.
6) Add 20 ml of compound (carcinogen) at desired concentration in complete MEM-E/Na medium containing 10% FBS to each roller flask and incubate at 37°C for 1.5 hours.

7) Aspirate compound off and wash with 5 ml complete MEM-E/Na.

8) Add 50 ml of complete MEM-E/Na medium (10% FBS) to each culture flask. Allow cells to grow an additional 6 days at 37°C.

9) Harvest cells by trypsinization, pellet by low speed centrifugation (1000 RPM for 10 minutes), and store frozen at -80°C.

Following is a flow chart which clarifies and condenses the cocarcinogenesis transformation format.

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**Co-Carcinogenesis Transformation of D Cells**

40 Roller Flasks (50-80% Confluent)

- **Pre-Infection**
  - Chemical
  - 1.5 hours.
  - Wash (MEME + 10% FCS)
  - DEAE-Dextran (40 g/ml)
  - 20 minutes
  - FeSV(FeLV)-ST
  - 6.10 x 10^3 FFU/Flask
  - 2 hours.
  - Chemical
  - 1.5 hours.

- **Post-Infection**
  - Feed (MEME + 10% FCS)
  - Harvest Cells
  - Pellet by Centrifugation
  - Freeze at -80°C
2) Purification of Nuclear and Cytoplasmic DNA From D 550 Cells Used in Cocarcinogenesis Transformation Studies or Which Served as ST-FeSV Infected of Non-infected Controls. 

a) Separation of Nuclear and Cytoplasmic Fractions From Human Fibroblast Cells. (Methods based on Ref. 44.)

The cell pellet (approximately 4-5 ml in volume) was suspended in 30 ml of hypotonic buffer consisting of 0.01 M Tris-HCl, pH 7.4, 0.01 M NaCl, and 0.003 M MgCl₂. The cells were allowed to swell for 20 minutes. Ten ml of a mixture of 6.7% Tween 40 (v/v) and 3.3% Na deoxycholate (w/v) 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 minutes at 4,000 RPM in a Sorvall SS34 rotor. The cytoplasmic supernatant was decanted and saved. The nuclei were resuspended in hypotonic buffer and recentrifuged (10 minutes 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 again 1.5 ml of a 6.7% Tween 40 (v/v) and 3.3% sodium deoxycholate (w/v) was added. This suspension was vortexed for 2-3 seconds and the nuclei were sedimented a third time (10 minutes at 4,000 RPM). A fourth resuspension in hypotonic buffer and resedimentation of the nuclei was done. This freed the nuclei of all traces of cytoplasmic material. The four cytoplasmic fractions were combined and frozen at -80°C.

b) Extraction and Purification of High Molecular Weight Nuclear DNA from D 550 Human Fibroblast Cells. (Methods were modifications of those reported in References 14 and 41.)

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 (0.01 N Tris-HCl, 0.1 M NaCl, and 0.001 M EDTA).

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 minutes) at 1000 µg/ml was added (500 µg/ml final concentration) and the mixture was incubated at 37°C for 2 hours.
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 minutes periods.

5) The phases were separated by centrifugation at 2,000 RPM for 15 minutes. 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 and one-tenth volume of 2 M Na acetate, pH 5.0 followed by storage for approximately 48 hours 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. At this time the DNA was sonicated on ice for 1 minute using 3 separate 20 second sonications. The sonication enhanced solubility of the DNA and reduced the size length to allow for increased efficiency of hybridization.

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

9) The RNase treated DNA was then digested with nuclease-free pronase at a final concentration of 10 µg/ml for an additional 2 hours at 37°C.

10) RNA present in the mixture was then hydrolyzed by heating at 65°C for 10 minutes with 0.25 M NaOH.

11) Following 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 and one-tenth volume of 2 M Na acetate, pH 5.0, followed by storage at -20°C for 48 hours.

13) The nuclear DNA was collected by centrifugation for 2 hours at 6,000 RPM using the Sorvall HB-4 rotor in a Sorvall centrifuge at 0°C and resuspended in a small volume of 1X TE buffer pH 8.0 (0.010 N Tris-HCl, 0.001 M EDTA).
14) The DNA was quantitated by measuring its optical density at 260 nm (50 μg 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. The DNA quantitation was confirmed by a second method which involved diluting a small quantity of it and mixing it with a known concentration of ethidium bromide. The fluorescence was then compared to that of a known dilution series of calf thymus DNA standards, which had been mixed with a similar concentration of ethidium bromide (68).

c) Purification of Cytoplasmic DNA from D 550 Human Fibroblast Cells

The methods used for purification of cytoplasmic DNA were essentially the same as those used of nuclear DNA with the following modifications.

1) In order to reduce the large volume of the cytoplasmic fractions (approximately 100 ml per 40-490 cm² roller bottles) and to avoid using tremendous quantities of Proteinase K for digestion, the cytoplasmic fraction was initially extracted at room temperature with 2 volumes of TNE saturated phenol: chloroform: isoamyl alcohol (25:24:1.) Usually one-tenth volume of 5% SDS in TNE, pH 8.0 was added prior to extraction to assist somewhat in breaking up the emulsified nature of the aqueous phase. The upper aqueous phase was separated by centrifugation at 2,000 RPM for 15 minutes and the DNA was precipitated at -20°C following the addition of 2 volumes of absolute ethanol and one-tenth volume of 2 M Na acetate, pH 5.0.

2) The precipitated DNA was collected by centrifugation at 6,000 RPM for 2 hours at 0°C in the GSA rotor of a Sorvall centrifuge. This DNA was resuspended in 20 ml of 1X TNE, pH 8.0 and at this time treated with an equal volume of Proteinase K at 1000 μg/ml (500 μg/ml final concentration) for 2 hours at 37°C. Sonication of cytoplasmic DNA was not necessary as it was already of sufficiently low molecular weight as to hybridize readily. Following extractions with TNE saturated phenol: chloroform: isoamyl alcohol (25:24:1) and precipitation at -20°C, the
cytoplasmic fractions were treated identically to the nuclear fractions including treatment with RNase A, pronase, and RNA hydrolysis. RNase A digestion was conducted at a final concentration of 100 µg/ml or twice that used for nuclear DNA due to the large amount of contaminating RNA in the cytoplasmic fractions. Quantitation was the same as that for nuclear DNA.

3) Propagation, Purification, and Radiolabelling of Plasmid DNA and the DNA of the v-fes gene of ST-FeSV.

a) Propagation of plasmid DNA.

E. coli strains D 5410 bearing pBR322 and K802 bearing cloned v-fes in pBR322 were both propagated in Luria-Bertani (LB) medium consisting of 10 gm Bactotryptone, 5 gm Bacto yeast extract, and 5 gm NaCl per liter. Magnesium chloride at 10 mM concentration was also routinely added. Tetracycline at 10 µg/ml was included in the selective medium used to grow the S_L or S_R subclones of v-fes (cloned in the ampicillin resistance gene) to exclude unwanted mutants, and ampicillin at 50 µg/ml was included in the LB medium used to propagate strain D 5410.

The E. coli strains were grown in 400 ml quantities in the dark at 37°C in shaker culture for 24 hours at which time the optical density was approximately 1.0 to 2.0 or until the cultures had reached log phase (approximately 4 x 10^8 cells/ml). At this time, chloramphenicol at 200 µg/ml was added to amplify plasmid DNA replication and shut down bacteriological chromosomal DNA synthesis (20). Incubation at 37°C was continued for an additional 24 hours prior to purification of plasmid DNA.

The various strains of E. coli were frozen down in LB medium plus 20% glycerol in liquid nitrogen for long term storage.

b) Purification of Plasmid DNA.

These methods were based on those used by Dr. John Reeve (The Ohio State University, Columbus, Ohio - Personal Communication) and on those presented by R. W. Davis et al. (27).

1) Approximately 1200 ml of culture fluid containing E. coli, bearing plasmid DNA, was clarified by centrifugation at 5,000 RPM for 20 minutes in the GSA rotor of a Sorvall centrifuge at 4°C.
2) The sedimented cell pellets were washed by resuspension in 40 ml of 25% sucrose prepared in 0.05 M Tris-HCl, pH 8.0.

3) The cell suspension was split and the cells were resedimented in each of 2 plastic centrifuge tubes (approximately 20 ml capacity) by centrifugation at 10,000 RPM for 10 minutes in the SS34 rotor of a Sorvall centrifuge at 4°C.

4) Each of the two cell pellets was resuspended in sufficient 25% sucrose in 0.05 M Tris-HCl, pH 8.0 to make a total volume 4 ml. (This was done directly in the plastic tubes used for sedimentation with a diameter of approximately 2.5 cm.)

5) 0.8 ml of lysozyme (Sigma) at 20 mg/ml (prepared fresh) in 0.25 M Tris-HCl, pH 8.0 was added to each tube and the mixture was swirled on ice for 5 minutes.

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

7) Finally, 6.4 ml of lytic mix consisting of 2% Triton X-100, 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 minutes on ice. If the mixture did not clear well, it was heated at 37°C for approximately 1 minute.

8) At this time a clearing spin was done to pellet large molecular weight chromosomal DNA from E. coli. The cleared lysates were centrifuged for 1.5 hours at 15,000 RPM and 4°C in the SS34 rotor of a Sorvall centrifuge. The clear supernatant containing plasmid DNA was carefully removed with the aid of a Pasteur pipet.

9) The volume of the supernatant, containing plasmid DNA was measured, and for every ml, exactly 2 gm of solid cesium chloride was added and the mixture was swirled gently until all salt was dissolved.

10) 0.8 ml of ethidium bromide solution (10 mg/ml in distilled H₂O) was added for each 10 ml of CsCl solution, and the preparation was mixed well and kept out of direct light. This procedure should result in a final density of 1.5 gm/ml (\( \eta = 1.380 \)) and the concentration of EtBr should be approximately 600 \( \mu \)g/ml.
11) The equilibrium density gradients were centrifuged for at least 48 hours at 41,000 RPM in the SW 50.1 rotor of a Beckman, Model LB-80 ultracentrifuge at 15°C.

12) The DNA containing bands were viewed by illuminating the tubes with long-wavelength UV radiation. The upper band contained linear, bacterial DNA and nicked, circular plasmid DNA. The lower band contained covalently closed circular plasmid DNA and was collected by puncturing the side of the tube with a 20 guage syringe needle.

   (Relaxed DNA: $\rho = 1.55$ gm/ml)

   (Native Superhelical DNA: $\rho = 1.59$ gm/ml)

13) Ethidium bromide was removed from the plasmid DNA in CsCl by adding 1 volume unit of isopropanol saturated with aqueous 5 M NaCl, 10 mM Tris, and 1 mM Na₂EDTA, pH 8.5. The mixture was shaken vigorously and the phases were allowed to separate on ice. The colored upper 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 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 hours 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 at 200 µg/ml (100 µg/ml final concentration) for 2 hours at 37°C.

17) The digested DNA was extracted by mixing with 2 volumes of TE-saturated phenol: chloroform: isoamyl alcohol (25:24:1) for 30 minutes at room temperature. The phases were separated by centrifugation at 2,000 RPM for 15 minutes and DNA was precipitated from the upper aqueous phase by addition of 2 volumes of cold, absolute ethanol and one-tenth volume of 2 M Na acetate, pH 5.0, and storage at -20°C for at least 48 hours.

18) This plasmid DNA was subjected to a second cycle of equilibrium density gradient centrifugation as described in steps 9-15 for further purification. DNA was finally dissolved in a small volume of 1X TE, pH 8.0 (100-200 µl) and stored at 4°C.
c) Restriction Endonuclease Digestion and Purification of v-fes DNA from the SL Subclone.

The restriction endonuclease digestion was based upon those procedures presented by T. Maniatis et al., (Ref. 68.). Restriction endonuclease digestion was conducted in a 1 ml reaction mixture.

1) 802 µl of sterile, distilled, nuclease-free H2O was placed in a nuclease-free microcentrifuge tube (1.5 ml capacity) along with 50 µg of pBR322 DNA containing the SL subclone of v-fes.

2) 100 µl of 10 X medium salt restriction endonuclease buffer (500 mM NaCl, 100 mM Tris-HCl, pH 7.5, 100 mM MgSO4, and 10 mM Dithiothreitol) was added and mixed on ice.

3) Finally, 50 units of Pst I restriction enzyme (Miles) was added. The tube was mixed by tapping and incubated at 35ºC for 1.5 hours.

4) The reaction was terminated by adding 20.4 µl of 0.5 M EDTA, pH 7.5 resulting in a final concentration of 10 mM.

5) The total mixture was extracted once with TE-saturated phenol: chloroform: isoamyl alcohol (25:24:1) and once with chloroform: isoamyl alcohol (24:1), each time with gentle stirring for 30 minutes at room temperature. The aqueous phases were collected by centrifugation at 2,000 RPM for 15 minutes.

6) DNA was precipitated from the final aqueous phase by addition of two volumes of absolute ethanol and one-tenth volume of Na acetate, pH 5.0 and storage at -20ºC for one to several days.

7) Precipitated DNA was sedimented by centrifugation at 6,000 RPM for 2 hours in the HB-4 rotor of a Sorvall centrifuge at 0ºC. Digested DNA was resuspended in 0.5 ml of 1X TE, pH 8.0 per 50 µg starting plasmid DNA.

8) Each 0.5 ml of digested plasmid DNA was mixed with 0.3 ml of ethidium bromide stock (10 mg/ml in H2O) and 0.4 ml aliquots of this mixture were layered onto 15-30% sucrose density gradients contained in SW 50.1 centrifuge tubes. Gradients were centrifuged for 5.25 hours at 15ºC in the SW 50.1 rotor of a Beckman ultracentrifuge, Model L8-80, at 40,000 RPM.
9) The top band containing cleaved v-fes DNA was carefully collected from above with a cannula type syringe needle or from the side by puncturing the tube with a 20-gauge syringe needle. The bottom, highly dispersed band consisted of nicked, linear pBR322 DNA. These DNA-containing bands were viewed by illuminating the tubes with long-wavelength UV radiation. This method of isolating the v-fes gene was used because of the difficulty often encountered in attempting to nick-translate DNA which has been purified by electrophoretic separation in agarose gels. The inhibitor of the DNA enzymes used in nick-translation is thought to be sulfonated carbohydrates present in the agarose and extensive purification must be done to rid the cleaved DNA from such contaminants (27).

10) Because isopropanol was miscible with the sucrose fractions collected from rate zonal gradients, ethidium bromide was removed by first adding cesium chloride to the sucrose fraction containing v-fes (1 gm/1 ml). This allowed for extraction of ethidium bromide in the isopropanol phase when 1 volume units of isopropanol (saturated with aqueous 5 M NaCl, 10 mM Tris, and 1 mM Na2EDTA, pH 8.5) were added to the DNA and mixed vigorously. Extractions were repeated until all visible color was removed. At this time 2 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.

11) The DNA was resuspended in approximately 25 µl of 1X TE, pH 8.0. Due to the small concentration of v-fes in the preparation, the concentration was determined using visual comparison to known quantities of calf thymus DNA standards which short-wave length UV background. Approximately 0.5 µg of v-fes DNA was recovered from 50 µg of starting plasmid DNA containing the SL subclone.

12) The purity of the v-fes DNA was checked by electrophoresing a small quantity in 1% agarose gels prepared and run in 10 mM sodium phosphate buffer, pH 7.0 for 1.5 hours at 45 mamps constant current. Comparisons were made to DNA from unrestricted pBR322-SL, pBR322, and from the lower band in sucrose density gradients containing linear
pBR322 DNA from which v-fes had been cleaved. In most cases a pure fraction of v-fes was recovered as evidenced by its migration as a single narrow band of low molecular weight in agarose gels. pBR322 has only one Pst I site into which the Pst I fragments of v-fes were cloned, hence digestion of a pure preparation of pBR322-SL with Pst I results in two fragments - one being linear pBR322 and the other being the SL subclone of v-fes DNA.

d) Nick-Translation of Plasmid and v-fes DNA.

This procedure was used as reported by Davis et al., (Ref. 27.).

This reaction was conducted in a final total volume of 25 μl. The following reagents were added in order to a capped, nuclease-free, 1.5 ml capacity microfuge tube.

1) 13.1 μl of 1X TE buffer, pH 8.0.
2) 2.5 μl of 10X Nick Translation buffer (0.5 M Tris-HCl, pH 7.5, 0.1 M MgSO₄, 10 mM Dithiothreitol or DTT, and 500 μg/ml of Bovine Serum Albumin or BSA).
3) 2.5 μl of a solution containing 0.2 mM each dATP, dGTP, and dTTP (Calbiochem-Behring).
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) Stock solution consisted of DNase at 1 mg/ml in 50 mM Tris (pH 7.5), 10 mM MgSO₄, 1 mM DTT, and 50% glycerol stored at -80°C.

b) 0.5 μl of DNase shock 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). Dilution was conducted at 0°C.

c) 0.5 μl of the above 1X diluted stock was diluted a second time to 100 μl in 1X nick translation buffer for use in the nick translation reaction (total dilution 1/40,000). This second dilution was also conducted at 0°C and the 2X diluted DNase was stored in small aliquots at -80°C.
6) To the above reaction mixture on ice, was added 5 µl of deoxy-
    cytidine 5'-α-32P triphosphate in aqueous solution containing 10
    µCi/µl. (Specific activity was 2,000-3,000 Ci/mmol, Amersham
    Corporation).

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

The above mixture was incubated at 14°C for 3 hours, and the reac-
    tion was stopped by addition of 25 µl of 0.02 M Na2EDTA, 2 mg/ml of
    sonicated salmon sperm DNA as carrier, and 0.2% SDS.

The mixture was placed on ice at this point 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) column (Bio-Rad Econo-column), pre-equilibrated
    with 1X TE, pH 8.0.

Ten to 12 effluent samples were serially collected in polypropylene
    microfuge tubes. The labeled v-fes or plasmid DNA usually eluted in
    tubes 9-12. The location of the 32P-labeled DNA was determined by
    spotting 2 µl samples of each fraction on 3 MM filter paper and counting
    in BBOT (Packard) cocktail in a Beckman liquid scintillation counter.
    The contents of the tubes comprising the first peak of 32P activity
    were combined and stored at 4°C in a polypropylene tube housed in a lead
    container.

    The specific activity of the labeled probe was generally between 7
    to 10.0 x 10^7 dpm/µg of DNA.

4) Dot-Blot Hybridization of Human Fibroblast DNA Infected with ST-FeSV
    to Purified v-fes or Plasmid (pBR322) DNA.

    a) Hybridization of DNA Using Formamide.

        This procedure was used routinely.

        1) GeneScreen hybridization transfer membrane (New England Nuclear-
            DuPont) was cut into approximately 5 x 6.5 inch sheets and gently laid
            onto 0.025 M Na2HPO4/Na H2PO4, pH 6.5 and soaked for 20-30
            minutes.
2) The GeneScreen membrane was then laid between two sheets of 3 MM chromatography paper (Whatman) and allowed to dry at 37°C.

3) Purified cellular DNA of known concentration was denatured by placing it in a water bath at 90°C for 10 minutes. Doubling dilution series of the denatured cellular DNA were immediately prepared in 1X TE, pH 8.0 on ice. Ten µl samples of each dilution were spotted in a predetermined pattern on the GeneScreen membrane, with a pipetman (Rainin). Spotting 10 µl by hand results in approximately a 1 cm diameter spot. (Approximately 20 spots or dilutions were placed onto a membrane of 5 x 6.5 inch dimensions.)

4) The spotted membrane was allowed to dry at room temperature and the DNA was fixed by baking it onto the membrane at 80-100°C for 2-4 hours in a drying oven (Thelco, Model 15, Precision Scientific Company).

5) The membrane was prehybridized by treating it with the following solution: 50% formamide (deionized)\(^1\), 2 x SSC\(^2\), 0.05 M 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), 0.02% ficoll (mol. wgt. 400,000) (Sigma), and denatured salmon sperm DNA (200 µg/ml) (Calbiochem-Behring.\(^3\) The membrane was placed into a sealable plastic pouch (Karak/Scotchpak) along with 40 ml of the solution and the plastic bag was sealed (Scotchpak pouch sealer) and incubated in a water bath with constant agitation overnight at 42°C.

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1 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 minutes at room temperature, filtered to remove resin, and stored in 10 ml aliquots at -20°C.

2 2X SSC: 0.3 M Sodium chloride
   0.3 M sodium citrate.

3 Salmon sperm DNA was denatured in water by heating at 90-100°C for 10 minutes.
6) After 12 hours, the prehybridization solution was removed from the plastic bag and replaced with 12 ml of the following solution: 50% formamide (deionized), 2X SSC, 0.05 M Na₂HPO₄/NaH₂PO₄, pH 6.5, 0.02% polyvinyl-pyrrolidone (mol. wgt. 40,000), 0.02% bovine serum albumin, 0.02% ficoll (mol. wgt. 400,000), denatured salmon sperm DNA (200 µg/ml) plus 12 x 10⁶ dpm of ³²P-labeled v-fes or plasmid probe which was denatured at 90°C for 10 minutes (approximately 1 x 10⁶ dpm was used per 1 ml of hybridization solution). The membrane and solution were double-bagged and sealed and incubated an additional 48-50 hours in a water bath with constant agitation at 42°C.

7) The hybridization solution was disposed of in the radioactive waste and the membrane was first washed two separate times for 5 minutes each with 100 ml of a solution containing 0.3 M sodium chloride, 0.06 M Tris-HCl, pH 8.0, 0.002 M EDTA. Washing was at room temperature with constant agitation.

8) The membrane was then washed 2 separate times for 30 minutes each with 100 ml of a solution containing 0.3 M sodium chloride, 0.06 M Tris-HCl, pH 8.0, 0.002 M EDTA, and 0.5% SDS. Washing was at 60°C with constant agitation.

9) Finally, the filter was washed two separate times for 30 minutes each with 100 ml of 0.003 M Tris base. Washing was at room temperature with constant agitation.

10) The membrane was then dried at room temperature and used in the autoradiography procedures described in section 4. c) which follows.

b) Hybridization of DNA Using Dextran Sulfate as a Hybridization Enhancer.

This procedure was used experimentally and not on a routine basis.

1) The membrane was prehybridized by treating it with 10 ml of the following buffer: 50% formamide (deionized), 0.2% polyvinyl-pyrrolidone (mol. wgt. 40,000), 0.2% bovine serum albumin, 0.2% ficoll (mol. wgt. 400,000), 0.05 M Tris-HCl, pH 7.5, 1 M 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).
Prehybridization was conducted in a sealed plastic bag which was placed in a water bath with constant agitation at 42°C overnight.

2) Following prehybridization, 2-3 ml of the following solution was added to the bag containing the prehybridization buffer and membrane: 50% formamide (deionized), 0.2% polyvinyl-pyrrolidone (mol. wgt. 40,000), 0.2% bovine serum albumin, 0.2% ficoll (mol. wgt. 400,000), 0.05 M Tris-HCl, pH 7.5, 0.1% sodium pyrophosphate, 0.1% SDS, denatured salmon sperm DNA (100 µg/ml) plus 12 x 10^6 dpm of 32p-labeled plasmid or v-fes probe which was freshly denatured at 90°C for 10 minutes.

This mixture was double bagged and sealed and incubated in a water bath with constant agitation for 48 hours at 42°C.

3) Washing and drying of the membrane was identical to that described in steps a) 7-10 of the preceding hybridization procedures using formamide.

c) Autoradiography of Hybridized DNA on GeneScreen Hybridization Transfer Membranes.

These procedures were based on those of Dr. H. G. Kung, Dept. of Biochemistry, Michigan State University, East Lansing, Michigan - Personal Communication.

1) After hybridization and drying, the membranes were placed between Saran wrap.

2) Kodak X-Omat AR film (XAR-5) was exposed to the membrane for 24 to 48 hours at -80°C. The intensity of exposure was amplified with the use of Dupont Cronex Lighting-Plus 2C intensifying screens without blockers.

3) Following exposure the film was developed using the following procedure:

   5 minutes in Kodak liquid X-Ray developer.
   1 minutes in 3% acetic acid stop bath.
   10 minutes in Kodak rapid fixer.
   15 minutes in running water.
4) The developed X-Ray films were allowed to dry at room temperature and the relative intensity of the spots resulting from exposure to the bound radioactive probe DNA on the membranes, was used to quantitate numbers of v-fes present in the human DNA screened.

d) **Quantitation of Genome Numbers of v-fes Present in Human Fibroblast Nuclear DNA.**

1) Initially a standard series of known copy numbers of v-fes were prepared in a background of denatured salmon sperm DNA (Calbiochem-Behring).

Mammalian diploid nuclear DNA is known to have an average molecular weight of $4 \times 10^{12}$ daltons (43). The $S_L$ subclone of v-fes is about 500 base pairs long or $0.33 \times 10^6$ daltons in molecular weight. The v-fes DNA was not restricted enzymatically for preparation of the copy standards, such that pBR322 plasmid DNA was also a portion of the background DNA.

As an example, for preparation of 100 $\mu$g of DNA containing 100 copies of v-fes per average diploid genome, the procedure was as follows:

\[
S_L = \text{approximately 500 base pairs (bp) or } 0.33 \times 10^6 \text{ daltons (d)} / (1000 \text{ bp} = 6.6 \times 10^5 \text{ daltons, for duplex DNA, Ref. 27})
\]

pBR322 containing the $S_L$ subclone = 4,362 bp + 500 bp = 4,862 bp.

\[
S_L = \frac{500 \text{ bp}}{4,862 \text{ bp}} = 0.1028 \text{ of the total of pBR322 and } S_L \text{ present in the mixture.}
\]

Hence, for every 1 $\mu$g of $S_L$, there exists $1/0.1028$ or $9.7276 \mu$g of pBR322 DNA as background DNA.

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

100 copies of $S_L = \frac{33 \times 10^6 \text{ d}}{4 \times 10^{12} \text{ d}} = \frac{1}{12000000} \times 100 \mu$g

1 diploid mammalian genome = $4 \times 10^{12} \text{ d}$

100 copies of $S_L = 8.25 \times 10^{-4} \mu$g per 100 $\mu$g total DNA.

pBR322 makes up $\frac{0.000825}{0.1028}$ or $0.008025 \mu$g of background DNA.
Hence, preparation of 100 μg of DNA containing 100 copies of SL (v-fes) per diploid mammalian genome required 0.00885 or 8.85 x 10^{-3} μg of pBR322 DNA containing SL plus 99.99115 μg of denatured salmon sperm DNA.

A dilution series of copy number standards was prepared to represent from 300 copies down to 1 copy per mammalian diploid genome. These copy standards were spotted in 10 μl amounts containing 100 μg of DNA each and compared to human fibroblast DNA for their relative hybridization to 32p-v-fes probe. Comparisons were made visually by the following method:

Doubling dilutions of human fibroblast DNA had been spotted onto the membranes in 10 μl quantities prior to hybridization such that a series of relative exposure intensities resulted on the X-Ray film. Comparisons were made where the intensity of the hybridized human nuclear DNA matched that of one 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

\[ \frac{100}{20} = 5 \times 10 \text{ or } 50 \text{ copies.} \]

Visual determination was semi-quantitative in nature and was sufficient for the low copy numbers of v-fes observed (see results). The method can be made more quantitative through the use of a densitometer.

2) The use of the prepared copy standards using cloned SL and salmon sperm DNA was discontinued when it was discovered that v-fes could not be cleaved from pBR322 and purified sufficiently to completely eliminate all sequences with some homology to pBR322 (Dr. Mariano Barbacid, NCI, Bethesda, Maryland - Personal Communication). See results III. B) section 1.

Hybridization of 32p-v-fes to the copy standards was in excess of that specific for v-fes because of background homology between the pBR322 sequences in the copy standards and in the probe which we were...
unable to remove totally. For this reason it was decided to use normal, uninfected human D 550 fibroblast DNA as a standard of comparison for genome enumeration. It recently has been found that the v-fes gene of ST-FeSV has homologous cellular sequences referred to as c-fes in all vertebrate species, including humans (40). The human c-fes locus has been localized on human chromosome 15 (25, 52). It has recently been sublocalized to the distal end of the long arm of chromosome 15 within bands q 25-26 by in situ hybridization of human c-fes probe to human mitotic chromosome preparations (51).

The human c-fes locus includes both coding and intervening sequences. It spans about 3.4 kilobases, with all three introns located in the 5' half of the c-fes gene. The human c-fes clones also appear similar in complexity to the cat c-fes locus (40).

It was known, therefore, that normal human DNA possesses a background of 2 copies of c-fes per diploid genome which hybridized to our $^{32}\text{P-v-fes}$ probe. All comparisons of DNA from chemically treated and ST-FeSV infected - D 550 cells were made with uninfected D 550 DNA as described for the copy standards in section III, 4) d) - 1.) This required that equal volumes of DNA of known concentration were spotted on the transfer membranes.

B. Results and Discussion of Hybridization Studies

1) Restriction endonuclease digestion of the $S_L$ subclone of v-fes in pBR322, with Pst I, did not result in a totally pure preparation of v-fes DNA. The low molecular weight species resulting from this digestion was collected from 15-30% sucrose density gradients and assayed for purity on 1% agarose gels following electrophoresis for 1.5 hours at 45 mAmps constant current in 10 mM Na$_2$HPO$_4$/Na H$_2$PO$_4$, pH 7.0.

Electrophoresis and staining with acridine orange appeared to indicate that the v-fes DNA was pure. It migrated as a single, narrow band of low molecular weight. The remainder of the digested DNA which consisted of linear pBR322 also migrated as a single band. However, there was some indication that a portion of the pBR322 DNA may have been
cleaved along with v-fes. The pBR322 DNA cleaved from the SL subclone ran at a slightly faster rate than the linear DNA in a commercial preparation of pBR322 DNA (Bethesda Research Laboratories). This indicated that it was of a lower molecular weight than linear pBR322 should be. Suspecting the possibility that our \( ^{32}p \)-labeled v-fes probe might contain some pBR322 DNA as a contaminant, hybridization to a pure commercial preparation of pBR322 DNA (Bethesda Research Laboratories) was conducted. Hybridization to 5.9 \( \mu g \) of pBR322 DNA/10 \( \mu l \) spotted, resulted in intense exposure of the X-ray film. Copy standards and uninfected human D 550 DNA were also run as controls.

It was apparent that our v-fes probe, despite our best efforts at purification, did possess some homology to pBR322. For this reason, use of standard copy series prepared using the SL subclone of pBR322 and salmon sperm DNA was of no real use, as the apparent copy number would be enhanced due to extraneous hybridization between pBR322 sequences in the probe and copy standards.

2) It was of interest to determine whether any homology existed between pBR322 plasmid DNA and human fibroblast DNA, particularly since we knew that our v-fes probe possessed some pBR322 sequences which could serve to enhance the hybridization signal.

Covalently closed, supercoiled pBR322 DNA was purified from E. coli strain D 5410. This DNA was separately treated with RNase A and pronase, and was extracted with TE-saturated phenol: chloroform: isoamyl alcohol (25:24:1) following isolation from CsCl equilibrium density gradients. pBR322 DNA was nick-translated and used as a hybridization probe with the following DNA preparations:

- Salmon sperm (Calbiochem-Behring) at 100, 50, 25, and 12.5 \( \mu g \) per 10 \( \mu l \).
- Calf thymus (Sigma) at 100, 50, 25, and 12.5 \( \mu g \) per 10 \( \mu l \).
- Human D 550 normal fibroblast at 56, 28, 14, and 7 \( \mu g \) per \( \mu l \).
- E. coli (Calbiochem-Behring) at 100, 50, 25, and 12.5 \( \mu g \) per 10 \( \mu l \).
- and pBR322 DNA (Bethesda Research Laboratories) - duplicates at 2.94 \( \mu g \) per \( \mu l \).
Hybridization was conducted for approximately 48 hours and the X-ray film was exposed to the membrane for 24 hours.

Results showed absolutely no homology between $^{32}\text{P}-\text{pBR322}$ and human, calf thymus, or salmon sperm DNA. All four dilutions of $E$. coli DNA and the pBR322 DNA showed obvious homology. The homology to $E$. coli DNA was most likely due to contaminating traces of $E$. coli nicked chromosomal DNA which equilibrated with pBR322 DNA in CsCl gradients. These results made it clear that any contaminating pBR322 sequences on $^{32}\text{P}-\text{v-fes}$ probe should not present background problems when used in hybridization experiments to human nuclear DNA.

3) An attempt was made to block out background homology to pBR322 DNA in prepared copy standards to make them suitable for genome enumeration. The copy standards had been prepared using a predetermined amount of v-fes DNA in pBR322 and denatured salmon sperm DNA. The traces of pBR322 present on the $^{32}\text{P}-\text{v-fes}$ probe contributed to background hybridization to the standards in addition to that specific for v-fes. Therefore their use as standards of comparison to human DNA for enumerating copy numbers of integrated v-fes resulted in inaccuracies. We hoped to circumvent this problem by prehybridizing the copy standards with pBR322 DNA to block background homology and subsequently to follow with hybridization to a $^{32}\text{P}-\text{v-fes}$ probe.

Following prehybridization overnight, the membranes containing copy standard DNA were hybridized to pBR322 DNA for 24 hours. A 100-fold excess of pBR322 over that contained in the spotted copy standards was used. The membranes were then washed in two separate changes of 0.025 M NaH$_2$PO$_4$, pH 6.5 to remove traces of pBR322 used in the initial hybridization. The copy standards were then hybridized with $^{32}\text{P}-\text{pBR322}$ (1 x $10^6$ dpm/ml hybridization buffer) for approximately 60 hours at 42°C. The membranes were extensively washed using three different solutions as described in methods Section III. 4a).

The X-ray film was exposed to the membranes for 45 hours. Blocking of background homology to pBR322 DNA was effective from 1 copy up through 25 copies of v-fes in the copy standards. Background homology
was not completely blocked in the copy standards prepared using 50 to 300 copies of v-fes. Attempts to perfect blocking of background homology to pBR322 in copy standards were no longer pursued when it became apparent that normal D 550 diploid DNA containing two copies of c-fes per genome could be used as our standard of quantitation.

It was felt that the blocking of pBR322 DNA in the copy standards could have been inadequate for several reasons. To begin with, there may not have been a sufficient excess of pBR322 DNA to block all sites of homology during prehybridization. Washing of the membranes following prehybridization to cold pBR322 DNA may have been inadequate and the membranes were not allowed to dry prior to final hybridization to 32p-pBR322. This may have created a sandwich effect whereby cold pBR322, which had only partially annealed to the copy standards or which had non-specifically absorbed to the membrane (as indicated by a large amount of nonspecific binding of the probe), was not adequately washed away and hybridized readily to 32p-pBR322 DNA.

4) A comparison was made of the efficiency of various 32p-v-fes probes in detecting v-fes homology in human DNA. Initial attempts at detecting v-fes homology in human DNA were made using the SL subclone of v-fes as a radiolabeled probe. For these experiments, v-fes was not cleaved out of pBR322 plasmid DNA. At no time were we ever able to observe hybridization of this probe with human DNA.

When v-fes was enzymatically restricted from pBR322 DNA and purified in rate zonal sucrose density gradients it became much more specific in its capacity to detect v-fes homology in human DNA. The two copies of c-fes present in normal diploid human DNA were always readily detectable using this 32p-v-fes probe. On occasion, when v-fes was not recovered from sucrose gradients completely free from restricted pBR322 DNA (as indicated by a second band in agarose gels), its efficiency as a probe for v-fes in human DNA decreased with its degree of contamination with pBR322 DNA.

For detecting low copy numbers of v-fes in our experimental and normal human DNA, it became crucial that our 32p-v-fes probe be as
free of contaminating pBR322 DNA as possible to increase specificity of hybridization. It has been reported by K. D. Rodland et al. (76) that a critical element in the success of hybridization reactions, using DNA immobilized on GeneScreen, is the purity and specificity of the labeled 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. We chose to use rate zonal sucrose density gradient centrifugation as opposed to electrophoresis for final purification to avoid contaminating compounds in agarose which often affect the efficacy of enzymes used in nick-translation.

5) Dextran sulfate was used experimentally in an attempt to enhance hybridization of $^{32}$P-uncleaved v-fes (v-fes-pBR322) to human nuclear DNA. Such an approach would have been useful in that restriction endonuclease digestion of v-fes-pBR322 and further purification v-fes would not have been necessary. Much more DNA would also have been available for use in nick-translation.

Dextran sulfate was included in both the prehybridization and hybridization solutions as described in methods, Section III. A) 4b). Non-specific background hybridization became a tremendous problem with this method. The unrestricted probe showed no evidence of hybridization to normal human DNA and was not specific in detecting the 2 copies of c-fes present in the human DNA.

It is known that the mass of the labeled 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 for us to use twice the recommended mass per volume of labeled probe in order to provide sufficient label for v-fes detection. This may have contributed to the problems with background binding on the membrane.

The use of both dextran sulfate and unrestricted v-fes DNA as probe in hybridization mixtures were rejected for the screening of experimental human DNA for v-fes homology. Best hybridization results were always obtained when v-fes was restricted from pBR322 vector DNA.
purified prior to nick-translation and when dextran sulfate was excluded from the hybridization solutions (See methods, Section III A) 4a).

6) A comparison was made between the amount of v-fes homology or genome copy numbers present in normal human D 550 DNA and in DNA from D 550 cells which had been infected with ST-FeSV (no chemical treatment). Nuclear DNA from three separate preparations of normal human D 550's containing 2 copies of c-fes per diploid genome, consistently hybridized to 32P-v-fes and was detectable on X-ray films when spotted at levels as low as 14 µg per 10 µl volume. Each DNA preparation was purified from a different batch of cells collected from 40-490 cm² tissue culture roller flasks. This provided a consistent quantitation standard for the chemically treated and ST-FeSV infected human DNA preparations which were screened.

Similar results were not obtained with ST-FeSV infected D 550 DNA. Again, three separate preparations of virus-infected D 550 DNA were screened numerous times for v-fes homology. Two of the three preparations showed no v-fes homology and one preparation showed background levels comparable to normal D 550 DNA when 20-40 µg were spotted per µl volume. We could not explain these results. We expected the D 550's which had been infected with ST-FeSV to integrate the provirus and show levels of homology above those seen in normal human DNA. This was not observed, however.

7) We screened the nuclear DNA from human D 550 cells, treated with chemicals at various times relative to ST-FeSV infection, for homology to v-fes DNA. None of the chemically treated D 550's showed levels of homology above that observed with normal, uninfected human DNA. Several of the treatments showed no detectable homology, similar to those findings observed with ST-FeSV infected human DNA. Normal, human DNA was included as a standard of comparison in all hybridization experiments. It always showed detectable levels of v-fes homology on X-ray films whereas some of the chemically treated and virus infected DNA did not.
Hybridization times used to screen the nuclear DNA for v-fes homology ranged from 48 to 66 hours and X-ray films were exposed to hybridized DNA for 48 to 72 hours.

Following are the hybridization results from the nuclear DNA of human D 550 cells exposed to various chemicals at 2 hours either previous to or post-ST-FeSV infection:

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Range of DNA Spotted (Doubling Level of Homology to v-fes)</th>
<th>DNA Spotted (Doubling Level of Homology to v-fes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) None Normal Diploid DNA</td>
<td>5.1 to 40.8 µg 2 copies per genome (All preparations tested)</td>
<td></td>
</tr>
<tr>
<td>2) ST-FeSV infected only</td>
<td>4.6 to 37 µg 2 copies per genome (1 out of 3 preparations only)</td>
<td></td>
</tr>
<tr>
<td>3) Hydrazine 2 hours before ST-FeSV infection</td>
<td>10.2 to 82 µg None detectable per 10 µl</td>
<td></td>
</tr>
<tr>
<td>4) Hydrazine 2 hours post ST-FeSV infection</td>
<td>10.5 to 84 µg None detectable per 10 µl</td>
<td></td>
</tr>
<tr>
<td>5) Mono-methyl Hydrazine 2 hours before ST-FeSV infection</td>
<td>3.3 to 26.2 µg 2 copies per genome per 10 µl</td>
<td></td>
</tr>
<tr>
<td>6) Mono-methyl Hydrazine 2 hours post ST-FeSV infection</td>
<td>9.2 to 73 µg 2 copies per genome per 10 µl</td>
<td></td>
</tr>
<tr>
<td>7) Symmetrical Dimethyl Hydrazine (SDMH) 2 hours before ST-FeSV infection</td>
<td>6 to 48 µg None detectable per 10 µl</td>
<td></td>
</tr>
<tr>
<td>8) Symmetrical Dimethyl Hydrazine (SDMH) 2 hours post ST-FeSV infection</td>
<td>5.3 to 42 µg None detectable per 10 µl</td>
<td></td>
</tr>
<tr>
<td>9) Unsymmetrical Dimethyl Hydrazine (UDMH) 2 hours before ST-FeSV infection</td>
<td>5.0 to 40 µg None detectable per 10 µl</td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td>Range of DNA Spotted (Doubling Dilution Series)</td>
<td>Level of Homology to v-fes</td>
</tr>
<tr>
<td>-----------------------------------------------</td>
<td>------------------------------------------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>10) Unsymmetrical Dimethyl Hydrazine (UDMH)</td>
<td>4.3 to 34 μg per 10 μl</td>
<td>None detectable</td>
</tr>
<tr>
<td>2 hours post ST-FeSV infection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11) Benzo(a) pyrene</td>
<td>6.3 to 51 μg per 10 μl</td>
<td>2 copies per genome</td>
</tr>
<tr>
<td>2 hours before ST-FeSV infection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12) Benzo(a) pyrene</td>
<td>5.3 to 42 μg per 10 μl</td>
<td>2 copies per genome</td>
</tr>
<tr>
<td>2 hours post ST-FeSV infection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13) Pyrene</td>
<td>2.3 to 18 μg per 10 μl</td>
<td>2 copies per genome</td>
</tr>
<tr>
<td>2 hours before ST-FeSV infection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14) Pyrene</td>
<td>2.8 to 22 μg per 10 μl</td>
<td>2 copies per genome</td>
</tr>
<tr>
<td>2 hours post ST-FeSV infection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15) Phenyl - α- Naphthylamine</td>
<td>7.9 to 63 μg per 10 μl</td>
<td>2 copies per genome</td>
</tr>
</tbody>
</table>

It was apparent that the chemicals used to treat the 550 human fibroblast cells did not enhance or reduce ST-FeSV proviral integration relative to cells which were only infected with ST-FeSV and which received no chemical treatment. Therefore, inhibition of transformation when carcinogens were applied at 2 hours post ST-FeSV infection could not be explained by differences in proviral DNA integration. The cytoplasmic DNA from each of the above listed chemical treatments has not yet been screened for relative levels of v-fes homology. This should provide valuable information about the ability of these chemicals to affect proviral DNA synthesis prior to integration into nuclear DNA.

The following section on final conclusions and discussion provides evidence that mechanisms other than proviral synthesis or integration could contribute to the decreased levels of transformation observed with chemical treatment.
IV. FINAL CONCLUSIONS AND DISCUSSION

Feline sarcoma virus is capable of highly efficient transformation of fibroblasts and epithelial cells in vitro. Transformation-specific proteins occur in these cells and appear to be unique for each individual strain of FeSV involved (7, 17, 18, 77, 92). The transformation-specific proteins associated with FeSV occur as fusion proteins, consisting of a portion of the FeLV core gene polyprotein (gag) linked to the protein encoded by fes, which is the onc gene responsible for transformation by ST-FeSV and Gardenr-Arnstein - FeSV (GA-FeSV) (21, 80, 83). Functional analysis of GA-FeSV and ST-FeSV gag-fes gene products has led to the identification of a protein kinase activity associated with each which has specificity for tyrosine residues (4, 73, 93).

Cellular transformation resulting from infection by the GA- or ST-FeSV strains leads to an increased level of total cellular phosphotyrosine. This presumably leads to the phosphorylation of critical substrates which are necessary for initiating and maintaining the transformed phenotype (4, 6, 74). It is possible in our experimental system that chemical treatment somehow interferes with the phosphorylation of critical viral or cellular substrates necessary for induction of the transformed state. The gag-fes polyproteins of GA- and ST-FeSV are functionally similar to the tyrosine kinases which are specific products of the transforming genes of several other retroviruses.

The transformation-specific polyproteins encoded by FeSV strains include 85,000 and 110,000 molecular weight polyproteins encoded by two different independent isolates of ST-FeSV (80, 83, 92), a 110,000 molecular weight polyprotein product of GA-FeSV (7, 92) and a 170,000 molecular weight product encoded by McDonough FeSV (7, 94).

The GA- and ST-FeSV polyproteins responsible for transformation contain FeLV p15 and p12 amino terminal structural components (7, 80, 83, 92) while the McDonough FeSV transformation-specific polyprotein, known as P a70gag-fms contains both of these proteins in addition to a FeLV p30 structural component (7, 94). In contrast to the polyproteins partially encoded by v-fes of GA- and ST-FeSV, the v-fms gene product,
represented within the McDonough FeSV-encoded polyprotein P
1709ag-fms, does not appear to possess intrinsic protein kinase
enzymatic activity (75, 96).

The 85,000 dalton polyprotein (P 85) of a variant of ST-FeSV pos-
sesses a single tyrosine acceptor site for its tyrosine-specific protein
kinase activity (12). Blomberg et al. (12) discovered that this accep-
tor site, as well as two serine phosphorylation sites localized within
the p12 structural component of ST-FeSV P 85, was phosphorylated in
cells nonproductively transformed by ST-FeSV. It is not known whether
phosphorylation of the tyrosine acceptor site in P 85 is of functional
significance in the transformation process. Other primary acceptor
sites of cellular origin may also be involved (12).

Reynolds et al. (74) have confirmed that there is reduced epidermal
growth factor (EGF) binding by GA- and ST-FeSV-transformed mink cells.
The significance of the close correlation between EGF binding and tyro-
sine-specific protein kinase activity is not known. Reynolds et al.
(74) feel that such a correlation could reflect the existence of tyro-
sine 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
(90) could be activated by phosphorylation of specific cellular tyrosine
acceptor sites and compete with EGF binding surface receptors. Although
a protein kinase activity has not been detected of the product encoded
by v-sis of simian sarcoma virus, it appears that this transforming
protein and a platelet-derived growth factor are derived from the same
or closely related cellular genes. In this case, v-sis may transform
cells by expressing a protein with functions similar or identical to
those of a normal cell growth factor, which is active during certain
phases of cell growth (28).

Nucleic acid homology has been detected between the v-fes gene of
GA- and ST-FeSV and v-fps which is the transforming gene common to the
Fujinami, PCR II, and UR I strains of avian sarcoma virus (49, 81). The
products encoded by v-fes and v-fps have also been found to be
immunologically and biochemically related (5, 8). Approximately 70% of the deduced amino acid sequences of the ST- and Ga-FeSV products are identical with residues in Fujinami sarcoma virus (FSV) P 130. All v-fes coding regions have a counterpart within the v-fps gene. The FSV polyprotein is larger than that of either ST- or GA-FeSV and includes additional v-fps encoded residues unique to FSV (50). It is apparent that the v-fes and v-fps sequences correspond to a common cellular genetic locus which has been highly conserved throughout vertebrate evolution. The tyrosine-specific protein kinase activities of their translational products have probable functional significance within their respective hosts (49). It also has been found that approximately 0.8 Kb at the 3' end of v-fes encode a protein domain with marked homology to the presumed carboxy-terminal end of the transforming protein (pp60scr) of Rous sarcoma virus. About 45% of the amino acids from the translated sequences from this region, are shared between the avian v-scr and the feline v-fes gene products (50). The amino acid homology with pp60scr has allowed for a predicted site of tyrosine phosphorylation within the ST-FeSV polyprotein. This site appears to be 7 residues to the carboxy-terminal side of a trypsin cleavage site and 5 residues to the carboxy-terminal side of a glutamic acid (TAC condon at position 2542) (50).

Extensive functional and structural homology between the predicted protein products of different v-onc genes provides strong evidence that the sequences which encode them may have evolved from a single ancestral gene or from a limited number of such ancestral genes. The v-fes, v-fps, and v-scr genes were derived from c-onc (cellular-onc) genes by independent recombination events involving different avian and feline leukemia viruses. It thus appears likely that different c-onc genes within the normal DNA of mammalian and bird cells may also be shown to be related (50). Limited homology such as that observed between pp60scr and the polyprotein encoded by v-fes might explained by exon shuffling whereby a segment of a tyrosine kinase gene was mobilized to a distal locus and placed under new transcriptional control (45).
<table>
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<tr>
<th>Acronym</th>
<th>Origin</th>
<th>Species of Isolation</th>
<th>Chromosomal Location of virally encoded protein</th>
<th>Activity of Virally Encoded protein</th>
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<td>9 2</td>
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<td>McDonough feline sarcoma virus</td>
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<td>5</td>
<td>Cytoplasm</td>
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(Table continued on next page)
MOLECULAR INTERACTIONS OF HIGH ENERGY FUELS AND JET FUELS WITH ONCOGENIC
OHIO STATE UNIV RESEARCH FOUNDATION COLUMBUS J R BLAKESLEE MAY 84
UNCLASSIFIED AFOSR-TR-84-0720 F49620-80-C-0087
Table of onc genes - continued

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<th>Acronym</th>
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<th>Chromosomal Location</th>
<th>Subcellular Localization</th>
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<td></td>
<td></td>
</tr>
<tr>
<td>rel</td>
<td>Reticuloendotheliosis virus</td>
<td>Turkey</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sis</td>
<td>Simian sarcoma virus</td>
<td>Woolly Monkey</td>
<td>22</td>
<td>15</td>
<td>Cytoplasm</td>
</tr>
</tbody>
</table>
Cellular homologs of several other retroviral transforming genes may in time prove to be members of a single gene family. Differences between the third base of the transforming gene \((v{-}yes)\) of avian Y73 sarcoma virus and RSV \(v{-}scr\) prevent their cross hybridization (57), but when the \(v{-}yes\) and \(v{-}scr\) genes were sequenced the predicted proteins were found to be very closely related (59). Thus the possibility arises that \(c{-}abl\) (Albelson murine leukemia virus) and \(c{-}ros\) (UR II avain sarcoma virus) may also represent divergent tyrosine kinase genes with segments related to those of other family members (50).

A family of proto-oncogenes also has been described which differs from the tyrosine-specific protein kinase gene family. This family includes the \(c{-}onc\) gene related to the \(v{-}ras\) sequences of Harvey and Kirsten murine sarcoma viruses, which code for distinct class of p21 ras transforming proteins (34). The \(v{-}ras\) encoded proteins have a guanosine diphosphate or guanosine triphosphate binding activity (60).

Of the approximately 18 isolations of acute transforming viruses whose \(onc\) genes have been distinguished, most can be grouped into families on the basis of molecular hybridization, comparison of primary structure, or comparison of the translated protein sequence. In addition to the relationship of the \(v{-}fes\) gene of GA- and ST-FeSV and \(v{-}fps\) of Pufinami sarcoma virus which has been discussed there are numerous examples of other \(onc\) gene relationships (47). The \(onc\) genes of the rat-derived Harvey sarcoma virus (\(has\)) and the mouse-derived BALB/c sarcoma virus (\(bas\)) are highly related. The newly described oncogene of Parodi-Irgens FeSV has shown a high degree of cross-hybridization uniquely with the \(sis\) oncogene of simian sarcoma virus. There appear to be more distant relationships between the \(ras\) family (consisting of \(has\) and \(bas\)) and \(kis\), the \(onc\) gene of rat-derived Kirsten sarcoma virus. The \(onc\) gene of RSV (\(src\)) has some relationship to \(fps\), \(fes\) and \(yes\) (\(onc\) gene of avian Y-73 virus) as mentioned earlier, as well as to \(abl\) and \(mos\) (Moloney murine sarcoma virus) and very distantly also to \(rel\) (avian reticuloendotheliosis virus) (47).
The cellular oncogenes have recently been grouped into three major gene families (src to raf; Ha-and Ki-ras; myc and myb) and a group of genes with no known homology to one another or to other known oncogenes (60). Each of these genes is presumed to be found in one or more copies in the genomes of all vertebrates, although this has not been documented for many of the genes (60).

It may be that the members of each onc gene family perform distinct but related functions. What these functions are and how they might be affected by chemical carcinogens are open for investigation.

The protein kinase activities of certain retroviruses have been characterized sufficiently to group them into three classes (82). They do not all function with equal efficiency under the same sets of conditions. The first class, of which RSV pp60src is an example, uses Mn+2 and Mg+2 with equal ability and has broad specificity for phosphate donors and nucleoside effector molecules. The second class, of which Abelson murine leukemia virus kinase is a member, prefers Mn+2 and Co+2 to Mg+2 and is capable of using GTP as a phosphoryl donor although at a much lower efficiency than ATP. The FeSV and Fujinami avian sarcoma virus kinases are members of the third class. This class prefers Mn+2, but has a rather strict specificity for ATP as a phosphate donor and for adenosine nucleotides as effectors. Because the translation product of Y 73 avian sarcoma virus is structurally unrelated to the protein kinase enzymes of the above classes, it may represent a fourth class (82). It is not known whether the above distinctions are significant in an overall hypothesis about protein kinase-mediated transformation or whether these differences merely reflect 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.

The transforming polyproteins of most retroviruses studied do not appear to have a common substrate or active site within their host cells (See table, pages 90 and 91). Rous sarcoma virus, pp60src, appears to
interact with plasma cellular membranes by means of an amino-terminal hydrophobic domain. Studies suggest that pp60^SRC is synthesized as a soluble protein which appears to undergo post-translational modification prior to association with the plasma membrane. Lipid binding and pp60^SRC plasma membrane association appear to be highly correlated leading to the suggestion that pp60^SRC may interact with the membrane by means of attachment to a fatty acid (42). The level of phosphotyrosine in the RSV-transformed cell is about 10-fold greater than that in the uninfected cell. However, it still makes up only a small proportion of phosphorylated protein residues which consist primarily of phosphoserine and phosphothreonine. It seems probable that only a limited number of protein substrates interact with pp60^SRC and lead to a cascade of reactions culminating in transformation cell. Vinculin has been found to be a specific substrate for pp60^SRC inside RSV-transformed fibroblasts (26). Vinculin is a structural protein generally involved in a type of linkage of the termini of microfilament bundles to normal cell membranes. It appears to concentrate at focal adhesion plaques formed between the cell surface and substratum and is specifically and closely apposed to several kinds of membrane sites at which microfilament bundles terminate. It has been proposed that phosphorylation of vinculin may in some way alter its function and affect the association of microfilament bundles with a decrease in cell substratum adhesion, thus, resulting in a rounding-up of the cells which is characteristic of RSV transformed-fibroblasts (26).

The routes leading to cell transformation appear to be quite diverse. The v-fms transforming gene of McDonough FeSV encodes molecules which do not appear to have protein kinase activity and whose properties and localization within cells differ distinctly from the products of other retrovirus oncogenes which have been characterized. The v-fms proteins are rapidly glycosylated and appear to be synthesized on membrane-bound polyribosomes. Multiple v-fms products exist and, because of substantial differences between newly synthesized and, steady-state products, it appears that multistep, post-translational processing
occurs within the endoplasmic reticulum-Golgi complex. These v-fms polyproteins appear to concentrate and remain associated with cytoplasmic organelles or cytoskeletal networks (1). These properties are quite different from those of v-src pp60, which is synthesized on "free" polyribosomes (66) and then becomes associated with the inner surface of the plasma membrane (24, 98); of v-ras p21, which only appears for a time in the cytosol and then becomes associated with the inner plasma membrane (99); and of the polyprotein encoded by v-abl, which spans the plasma membrane (100).

This contrast in mechanisms of transformation is further exemplified by three avian retroviruses which exhibit different oncogenic properties in vivo but all of which transform fibroblasts in vitro. The gag-myc protein of avian myelocytomatosis virus MC 29 is located in the nucleus and binds to DNA after purification. The gag-erb A protein of avian erythroblastosis virus (AEV) is not nuclear, but is probably located in the cytoplasm and does not bind to DNA after purification. Neither the gag-myc nor gag-erb A proteins exhibit protein kinase activity. In contrast, the gag-fps protein, encoded by Fujinami sarcoma virus, does have protein kinase activity but is not located in the nucleus and does not bind to DNA (71). P 130 of Fujinami sarcoma virus is present at some areas of the plasma membrane, but is widely distributed in the cytoplasm, which may suggest that its primary targets are also widely distributed in the cytoplasm. P 130 does not appear to be an integral membrane protein, but rather appears to interact with structural protein matrix (38). Because of similarities between P 130 and the transforming proteins of certain other retroviruses with protein kinase activity, Feldman et al. (38) have suggested that interaction with a structural protein matrix may be a general property of transforming proteins associated with tyrosine-specific protein kinase activities. Chemicals may somehow interfere in this interaction and circumvent or enhance the transformation process.

Since our particular study has been concerned with a loss of transforming activity by ST-FeSV in the presence of chemicals, it is of
interest that certain recent studies have found that loss of the transformed phenotype is associated with increased cytosine methylation of proviral DNA. Certain of the carcinogens used in our system are alkylating agents and, in particular, mono-methyl hydrazine and the symmetrical and unsymmetrical forms of dimethyl hydrazine are known to transfer methyl groups to certain regions of the DNA with which they interact.

Groffen et al. (48) demonstrated a correlation between methylation of cytosine residues and expression of the major ST-FeSV proviral DNA translational product in transformed mink cells. Increases in cytosine methylation were 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). When Groffen et al. (48) looked at both transformed and revertant clones, they found that, in contrast to v-fes proviral DNA, the c-fes (cellular homolog of ST-FeSV) transforming sequences were highly methylated in cytosine residues. Methylation of cytosine residues may represent an important level of regulation over gene expression involved in viral oncogene-mediated transformation and of the expression of cellular homologs of viral oncogenes.

Similarly, McGeady et al. (69) found that they could inhibit the transforming activity of cloned Moloney sarcoma virus (MSV) proviral DNA in vitro methylation of the DNA at cytosine residues. They concluded that methylation of the v-mos gene was more inhibitory to transformation than methylation of the viral long terminal repeat (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 murine
leukemia virus in murine embryonal carcinoma (EC) cells. Although infected EC cells contained up to 100 integrated proviral genomes, their expression was suppressed. This inactivation was correlated with de novo methylation of the viral DNA, which may be a characteristic of early embryonic cells (84).

In conclusion, the expectation should not be for a single common mechanism involved in retroviral transformation. In most cases, a multicomponent system probably controls growth and differentiation. Perhaps a few more generalizations may be made about the transforming proteins with tyrosine-specific kinase activity. In this case multiple membrane alterations may lead to a spectrum of activities related to the functioning of cytoskeletal elements and possibly second messenger functions (46). How chemicals may interact in the complex process of retroviral transformation has been much less studied. We can only speculate that the effects of chemicals will be largely determined not only by their individual modes of action, but by the mechanism of transformation utilized by the virus with which they interact.
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24. Courtneidge, S. A., Levinson, A. D., and Bishop, J. M. 1980. The protein encoded by the transforming gene of avian sarcoma virus (pp60src) and a homologous protein in normal cells (pp60proto-src) are associated with the plasma membrane. Proc. of the National Academy of Sciences, USA 77: 3783-3787.


CELLULAR SYSTEMS FOR TOXICITY TESTING 407
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ANNALS OF THE NEW YORK ACADEMY OF SCIENCES
INTRODUCTION

The utilization of a rapid in vitro assay to evaluate chemicals for carcinogenic activity would greatly reduce the time now required to determine carcinogenicity in animal test systems. The objectives of these studies were to determine whether chemicals altered ST-PeSV virus-directed transformation of human skin fibroblasts in a predictable manner and to correlate the alteration with carcinogenic or noncarcinogenic activity of the test chemical. The procedures used to study these interactions were described previously.1,2

The results, to date, showed that diverse classes of carcinogens inhibited virus transformation when virus-infected cells were exposed to test chemical two hours postinfection while noncarcinogens had no significant effect on transformation at two hours postinfection. Continued studies showed that the carcinogens inhibited a specific viral gene function, i.e., transformation, but not other viral gene products (feline oncornavirus—associated cell membrane antigen, group-specific antigen, and reverse transcriptase) that were shown to be cell mediated.1 The studies reported here expanded the classes of carcinogens and noncarcinogens tested to include hydrazines, jet fuels, asbestos, formalin, methylazoxy methanol acetate,4 and aromatic amines used as lubricant additives—alpha and beta naphthylamine and phenyl alpha and phenyl beta naphthylamine.

MATERIALS AND METHODS

Cells and Virus

Detroit 550, normal male human skin fibroblasts (HSFs) were grown under standard conditions as previously described.1 The Snyder-Theilen strain of feline sarcoma virus was prepared from feline embryo cell culture, titrated in HSFs, and stored at -85°C.

Transformation Assays

Preconfluent log phase growth HSFs were seeded into 16-mm diameter wells at 4 x 10⁶ cells/well in 1.5 ml complete medium and incubated 18 hours prior to...
Figure 1. Commercial formalin (37% HCHO–10% CH₃OH) was diluted in complete medium at 10 ppm and 1 ppm final concentration. Cells were treated and infected as described in Materials and Methods. Significance was determined by Student's t-test.
treatment in 5% CO₂ at 37°C. Cells were infected with 20 focus-forming units/well. Cells chemically treated prior to virus infection were exposed to chemicals for 30 minutes, washed, and refed until virus infection. Virus-infected cells were treated 30 minutes with chemicals, washed, and refed with complete medium. Seven to 10 days post infection, cells were fixed and stained with Giemsa and foci enumerated with a 25–40x dissecting microscope. Data were evaluated statistically by the Student "t"-test.

<table>
<thead>
<tr>
<th>Chemical Group</th>
<th>Reported Activity*</th>
<th>Inhibition of ST-FeSV Transformation</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Aromatic amines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. Naphthyl amines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Two (2)</td>
<td>C yes</td>
<td></td>
</tr>
<tr>
<td>2. Phenyl-alpha</td>
<td>C yes</td>
<td></td>
</tr>
<tr>
<td>3. Phenyl-beta</td>
<td>C yes</td>
<td></td>
</tr>
<tr>
<td>4. N-Acetoxy 2 fluorenylacetamide</td>
<td>C yes</td>
<td></td>
</tr>
<tr>
<td>II. Polycyclic Hydrocarbons</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Benzo[a]pyrene</td>
<td>C yes</td>
<td></td>
</tr>
<tr>
<td>2. Pyrene</td>
<td>NC no</td>
<td></td>
</tr>
<tr>
<td>III. Hydrazines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Hydrazine</td>
<td>C yes</td>
<td></td>
</tr>
<tr>
<td>2. Mono-methyl hydrazine</td>
<td>C yes</td>
<td></td>
</tr>
<tr>
<td>3. 1,1 Dimethyl hydrazine</td>
<td>C yes</td>
<td></td>
</tr>
<tr>
<td>4. 1,2 Dimethyl hydrazine</td>
<td>C yes</td>
<td></td>
</tr>
<tr>
<td>IV. Other</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Aflatoxin B₁</td>
<td>C yes</td>
<td></td>
</tr>
<tr>
<td>2. Amosite asbestos</td>
<td>C yes</td>
<td></td>
</tr>
<tr>
<td>3. JP5 (shale)</td>
<td>NC no</td>
<td></td>
</tr>
<tr>
<td>4. JP5 (petrol)</td>
<td>NC no</td>
<td></td>
</tr>
<tr>
<td>5. RJ5</td>
<td>NC no</td>
<td></td>
</tr>
<tr>
<td>6. Diesel fuel, marine</td>
<td>NC no</td>
<td></td>
</tr>
<tr>
<td>7. Acetone</td>
<td>NC no</td>
<td></td>
</tr>
<tr>
<td>8. Methyl azoxymethanol-acetate</td>
<td>C yes</td>
<td></td>
</tr>
<tr>
<td>9. Formalin</td>
<td>C yes</td>
<td></td>
</tr>
<tr>
<td>10. Triton X-100</td>
<td>? no</td>
<td></td>
</tr>
</tbody>
</table>

*Data from References 5-7.
+C, carcinogen; NC, noncarcinogen.

RESULTS

The data displayed in Figure 1 are typical of the results for chemical-treated virus-infected cells. Predetermined nontoxic doses of commercial formalin (37% HCHO–10% CH₂OH) at 10 ppm and 1 ppm were used. Ten parts per million significantly inhibited virus transformation by values ranging from 50% to 90% of control values, while 1 ppm affected virus transformation to a lesser degree. Although transformation was inhibited at four time periods for formalin, the results of our studies with over 20 different chemicals (Table 1) show that the time periods most
affected are two and six hours postinfection. The results shown in Table 1 are based on these time periods. The data compiled from this assay correlated well with the carcinogenic potential determined by the standard rodent assays.

REFERENCES

INDUCTION OF RETROVIRUS NON-PRODUCER HUMAN CELLS TO PRODUCER CELLS BY DEXAMETHASONE

JAMES BLAKESLEE, ANN ELLIOT AND DEBRA TURNER.
Department of Veterinary Pathobiology, 1925 Coffey Road, Columbus, OH 43210, U.S.A.

We previously reported human neonatal skin fibroblast cells treated with non-toxic concentrations of benzo (a) pyrene or aflatoxin B1, inhibited feline sarcoma virus (FeSV) transformation.1 Although comparable levels of reverse transcriptase (RT) activity were found in both chemically treated and untreated cultures, infectious virus was not recoverable from either.

Others have documented the stimulation and activation of murine and primate retroviruses and enhanced FeSV transformation of human cells by optimal concentrations of glucocorticoid hormones.2-4 The objectives of this study were to determine: 1) whether dexamethasone (DXM) induced both FeSV and FeLV synthesis in the apparent non-producer human cells and 2) whether feline oncornavirus-associated cell membrane antigen (FOCMA) and group-specific antigens (GSA) were synthesized in addition to RT, in non-DXM treated cells.

Snyder-Theilen strain FeSV4 infected Detroit 350 neonatal human skin fibroblast cells produced very low levels of transforming virus when supernatant fluids from these cultures were titrated in normal D550 cells. The addition of 1.0 µg/ml DXM (9α-Fluoro-16a-methyl prednisolone) 24 hours post infection, significantly increased both FeSV and FeLV5 synthesis when compared to non-DXM treated infected cells (Table 1). The increase in titer ranged from a 56-fold increase to a 100-fold increase for FeSV, while FeLV increased from 0 in untreated cultures to 6 X 106 FFU/ml in DXM treated cultures, a 2.6 fold excess when compared to FeSV.

RT activity (cpm of 3H dTMP incorporated/reaction/hr)1 likewise increased. The values ranged from a 9-fold increase in 1 experiment to a 11 fold increase in 2 other separate experiments.

GSA6 and FOCMA7 were detected in both FeSV infected DXM treated cells and FeSV infected cells only. Uninfected-cells, cell treated with 0.2% acetone, and/or DXM were negative.
TABLE I
DXM ENHANCED ST-FeSV AND FeLV SYNTHESIS IN D550 CELLS

<table>
<thead>
<tr>
<th>Virus and Exp #</th>
<th>Virus Titer (FFU/ml x 10^-2)</th>
<th>Fold Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-) DXM</td>
<td>(+) DXM</td>
<td></td>
</tr>
<tr>
<td>(1)</td>
<td>2.3</td>
<td>203.2</td>
</tr>
<tr>
<td>(2)</td>
<td>2.3</td>
<td>233.0</td>
</tr>
<tr>
<td>(3)</td>
<td>3.4</td>
<td>191.2</td>
</tr>
<tr>
<td>Untreated Control</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>FeLV^3</td>
<td>mean = 2.7 ± .3 mean = 209.2 ± 17.6</td>
<td>P = &lt; 0.001*</td>
</tr>
</tbody>
</table>

* = Determined by Students "t" test.

Wu et al. described retrovirus infected cells as falling into 3 phenotypic categories with respect to virus gene expression: (1) virus producer cells; (2) non-producer cells with partial gene expression (any virus, component); and (3) cells not having any detectable viral gene products. In this study, D550 cells infected with ST-FeSV could be classified as nonproducers with partial gene expression, i.e. transformation, reverse transcriptase, GSA and FOCIA antigens with little infectious virus being released. However, following DXM treatment, complete viral gene expression resulted with infectious (transforming) virus released.

The fact that DXM permits complete oncogenic viral gene expression in human cells is significant since the phenotypic expression of most human transformed cells has formerly been shown to be category 2 (non-producer) with partial gene expression.^8,9

ACKNOWLEDGEMENTS
Supported in part by a contract from AFOSR, F49620-77-C-011 and The Graduate School, The Ohio State University.

REFERENCES
4. Schaller et al. (1976), Cancer Res. 36, 1350-1357.
5. Heding et al. (1976), Cancer Res. 36, 1647-1652.
We previously described experiments in which chemical carcinogens significantly inhibited ST-FeSV transformation of human skin fibroblasts (1). The effect was not a result of cell killing because non-toxic dose levels were used. Further studies suggested the carcinogens inhibited a specific viral gene function or product (v-fes), whereas other viral gene products (gag, pol and F0C2h-S) were unaffected by chemical treatment as determined with the assay system used (2). These data suggested that this cell-virus system may be of value as a rapid in vitro assay to evaluate chemicals for carcinogenic activity. Diverse classes of carcinogens and non-carcinogens were assayed in order to determine if chemicals modified ST-FeSV transformation frequencies of human skin fibroblasts in a predictable manner, and to correlate the modification with the carcinogenic or non-carcinogenic activity of the test chemical.

The results, to date, showed carcinogens inhibited v-fes when virus-infected cells were exposed to test chemical 2 hrs. post-
infection (p.i.) while non-carcinogens had no significant effect on transformation at 2 hrs. p.i. For example, the aromatic amine N-acetoxy-2-fluorenyl acetamide inhibited v-fes transformation, whereas its non-carcinogenic analogue N-(4-fluorenyl) acetamide did not. Chemicals tested to date are shown in Table 1.

**TABLE 1**

<table>
<thead>
<tr>
<th>Chemicals Tested</th>
<th>(Modified v-fes Expression)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrazine</td>
<td>Phenyl(Naphthylamine)</td>
</tr>
<tr>
<td>Monomethyl hydrazine</td>
<td>N-Acetoxy-2-fluorenyl acetamide</td>
</tr>
<tr>
<td>1,1 Dimethyl hydrazine</td>
<td>Benzo(a)pyrene</td>
</tr>
<tr>
<td>1,2 Dimethyl hydrazine</td>
<td>JP5 (petroleum derived)</td>
</tr>
<tr>
<td>Aflatoxin B1</td>
<td>JP10 (petroleum derived)</td>
</tr>
<tr>
<td>Methyl Azoxymethanol-acetate</td>
<td>RJS</td>
</tr>
<tr>
<td>2-Naphthylamine</td>
<td>Diesel Fuel</td>
</tr>
<tr>
<td>Phenyl(a)Naphthylamine</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>(No Effect on v-fes Expression)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-(4-fluorenyl)acetamide</td>
</tr>
<tr>
<td>Pyrene</td>
</tr>
<tr>
<td>Anthracene</td>
</tr>
<tr>
<td>Phenanthrene</td>
</tr>
<tr>
<td>Benzo(e)pyrene</td>
</tr>
</tbody>
</table>

The data compiled in this assay correlated well with the carcinogenic potential as determined by rodent tumor assays (3). The temporal relationship between chemical treatment and virus infection suggests the chemicals intervene in v-fes synthesis or integration.

**REFERENCES**


Supported by AFOSR Contract F49620-80-C-0087.
IN VITRO EFFECTS OF POLYNUCLEAR AROMATIC HYDROCARBONS ON FeSV TRANSFORMATION OF HUMAN CELLS.

JAMES R. BLAKESLEE\(^1,2,3\), ANN M. ELLIOT\(^1\), LINDA J. CARTER\(^1\)
Department of Veterinary Pathobiology\(^1\), Department of Microbiology\(^1\), and the OSU Comprehensive Cancer Center\(^1\), 1925 Coffey Rd., Columbus, OH 43210, USA.

INTRODUCTION

A rapid in vitro assay to evaluate chemicals for carcinogenic activity would greatly reduce the time now required with animal test systems. Therefore, studies were undertaken to determine whether chemicals altered Snyder-Theilen feline sarcoma virus (ST-FeSV) directed transformation of human skin fibroblasts (HSF) in a predictable manner and to correlate the alteration with carcinogenic or non-carcinogenic activity of the test chemical. We initially reported that Benzo(a)-pyrene (BaP), N-acetoxy 2-fluorenylacetamide (A-AAF) or aflatoxin B1 (AFB1), inhibited virus-directed ST-FeSV transformation of HSF (1). It was further shown that the carcinogen treatment inhibited a specific virus gene function, i.e. transformation, but not virus synthesis. Other viral gene products, RNA-dependent DNA polymerase (RDDP), Group-Specific Antigens (GSA) and Feline Oncornavirus-Associated Cell Membrane Antigen (FOCM) were detected in both carcinogen-treated or non-treated virus-infected cells (2). These studies suggested the inhibitory effect on virus-directed transformation was mediated by the carcinogen treatment, while the inhibitory effect on complete virus synthesis was cell-mediated. Further, the inhibitory effect of the carcinogens was abrogated when chemicals were added to virus-infected cells 48 hours post-infection (p.i.). A time-dependent function was also observed which showed diverse classes of carcinogens inhibited virus transformation when virus-infected cells were exposed to test chemicals 2 to 6 hours post-infection, while non-carcinogenic chemicals had no significant effect on transformation (3).

As a continuation of these studies we correlated the effect of the following chemicals with ST-FeSV transformation:

1) 2-naphthylamine (NA)
2) phenyl alpha naphthylamine (PANA)
3) phenyl beta naphthylamine (PBNA)
4) petroleum-derived JP5 jet fuel (PD-JP5)
5) shale oil-derived JP5 jet fuel (SD-JP5)
6) 1,2 symmetrical dimethyl hydrazine (SDM)
7) methyl azoxymethanol (acetate) (MAA)
8) tetraethylthiuram disulfide (disulferam [DS])

MATERIALS AND METHODS

Cells

Human foreskin fibroblast cells (Detroit 550-CCL-109, American Type Culture Collection, Rockville, MD) were grown in Minimal Essential Medium with Earles salts supplemented with 1.0 mM sodium pyruvate, 2 mM glutamine, 1% non-essential amino acids, 50 μg/ml gentamycin (Schering Diagnostics, Port Reading, NJ) 0.22% sodium bicarbonate and 10% fetal bovine serum (Sterile Systems, Logan UT), thereafter designated Complete Medium (CM). Cells were serially passaged every 3-4 days at 1:2 split ratios and incubated at 37°C in 5% CO₂.

Virus

The preparation of stock ST-FeSV was described previously (4). Briefly, 10% cell-free tumor homogenates were prepared and frozen at -85°C in L15 medium and 5% FBS.

Transformation

Preconfluent log phase growth HSF cells were trypsinized and 4 x 10⁵ cells seeded onto 16 mm wells (Costar, Cambridge, MA) in 1.0 ml CM and incubated 18 hrs. prior to treatment. Cells pre-treated with chemicals prior to virus infection were incubated 90 min. with appropriate chemical concentration at 2, 6, or 24 hr. pre-infection. Cells were washed 2X in CM, refed and incubated at 37°C until virus infection. Cells to be infected were washed with serum-free CM and treated with 0.2 ml of DEAE-Dextran (40 μg/ml Sigma, St. Louis, MO) in serum-free CM. After 20 min., the cells were washed with CM + 5% FBS, infected with 0.05 ml ST-FeSV, diluted to 1,000 focus-forming units (FFU) per ml. Twelve wells were used for each time period. Plates were rocked at 10-15 min. intervals and virus adsorbed 2 hr. at 37°C. After adsorption, the inocula were aspirated and cells refed with 2.0 ml CM. Virus-infected cells were treated at 2, 6, or 24 hr. following virus adsorption by incubating infected cells with designated concentration of chemical for 90 min. followed by washing and refeeding cells with 2.0 ml CM. The cells were refed with CM on the 6th day p.i., and then
fixed with 10% phosphate-buffered formalin and stained with Giemsa 3-4 days later. Foci were counted at 25-40X magnification with a dissecting microscope in non-treated (control) and chemically-treated wells. FFU ± S.D. were determined for each treatment time and significance determined by Student's "t" test.

Toxicity Assays

Chemical toxicity was determined by seeding 8 wells with 500 cells per well as described for transformation assays. Cells were treated 90 min., washed and refed with CM + 20% FBS. Cultures were incubated 9-10 days, fixed, stained with Giemsa and clones containing at least 50 cells were counted. Toxicity was determined by dividing the average number of clones in treated wells by the average number of clones in control wells.

Chemicals

NA, PANA, PBNA, MNA and DS were dissolved in spectral grade acetone at 1.0 mg/ml. Prior to use, dilutions were made in CM to experimental concentrations. SDH was dissolved in 0.1 N HCl at a concentration of 10 mg/ml and diluted in CM to experimental concentrations.

Both petroleum or shale oil-derived fuels, JP5, diesel fuel, marine, RJ5 and JP10, were dispersed in serum-free CM with shaking. Dilutions tested were from 5,000 to 0.5 ppm. Eight wells, seeded with 500 cells/well, were treated for 90 min., washed and refed with CM supplemented with 20% FBS. Cultures were incubated 9-10 days, fixed in 10% buffered formalin, stained with Giemsa, and clones containing at least 50 cells were counted. Toxicity was determined by dividing the average number of clones in treated wells by the average number of clones in control wells. LD50 values were determined by plotting percent survival (ordinate) against concentration (abscissa, log scale) on 3-cycle semi-logarithmic graph paper. LD50's were then determined by inspection.

RESULTS

Petroleum-derived (PD) or shale oil-derived (SD) JP5 or diesel fuel, marine (DFM) cytotoxic analyses are shown in Table 1. The results show no significant difference in toxicity values between SD or PD fuels. LD50's for SD, JP5 and PD-JP5 were 102 ppm and 100 ppm, respectively, SD-DFM and
and PD-DFM were 85 ppm and 87 ppm, while LD_{50} s for JP10 and RJ5 were 91 ppm and 19 ppm, respectively.

TABLE 1
LD_{50} CYTOTOXICITY OF SHALE OIL AND PETROLEUM-DERIVED FUELS IN HSF CELLS.

<table>
<thead>
<tr>
<th>Fuel</th>
<th>Derived From</th>
<th>LD_{50}(ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>JP5</td>
<td>Shale</td>
<td>102</td>
</tr>
<tr>
<td>JP5</td>
<td>Petroleum</td>
<td>100</td>
</tr>
<tr>
<td>DFM</td>
<td>Shale</td>
<td>85</td>
</tr>
<tr>
<td>DFM</td>
<td>Petroleum</td>
<td>87</td>
</tr>
<tr>
<td>RJ5</td>
<td>Petroleum</td>
<td>19</td>
</tr>
<tr>
<td>RJ10</td>
<td>Petroleum</td>
<td>91</td>
</tr>
</tbody>
</table>

PANA treatment resulted in a dose-related suppression of transformation (Fig. 1A). Cells treated with 10 µg/ml resulted in significant inhibition of transformation at all time periods tested. Results with 20 µg/ml were similar, although treatment at 6 hrs. p.i. approached control values. Cells treated with 0.1 µg/ml showed no difference in transformation frequencies from untreated controls.

Treatment with 10 µg/ml PANA resulted in suppression at all time periods tested (Fig. 1B). Inhibition of transformation ranged from values of 40% to 65% inhibition. However, cells exposed to 0.01 µg/ml before ST-FeSV infection resulted in enhanced focus formation at -2 hrs., whereas significant inhibition was observed when virus-infected cells were treated 6 hrs. p.i.

Treatment with 50 or 5 ppm PD-JP5 at all time periods tested resulted in no differences in transformation frequencies from control values (Fig. 2). ST-FeSV transformation frequencies were significantly reduced when cells were treated with either 50 or 5 ppm SD-JP5 24 hrs. prior to virus infection, whereas transformation frequencies were similar to control values at the other time periods tested.
FIGURE 1. HSF cells were plated in 16 mm diameter wells with 2.0 ml CM and incubated overnight. Cells were treated as described in Materials and Methods. A-PANA, B-PENA and C-NA.
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FIGURE 2. Petroleum and shale oil-derived JP5 effects on ST-FeSV transformation. Assay described in Materials and Methods.

The biological specificity of the assay is shown by the data in Tables 2 and 3. Ten μg/ml SDM+ or 10 μg/ml SDM+ mixed with 0.01 μg DS were added to HSF 2 hrs. p.i. (Table 2). As shown in Table 2, SDM+ reduced the transformation frequency by 44% (p=0.001), whereas co-treatment with SDM+ + DS resulted in a non-significant 22% difference in the transformation frequency (p=N.S.). One ppm MAMA or 1 ppm

TABLE 2
ABROGATION OF THE INHIBITORY EFFECT OF SDM+ ON ST-FeSV TRANSFORMATION BY DS.

<table>
<thead>
<tr>
<th>Chemical Treatment</th>
<th>FFU/ml X 10^-1</th>
<th>% Reduction in FFU</th>
<th>(p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDM+</td>
<td>1.41</td>
<td>44</td>
<td>0.001</td>
</tr>
<tr>
<td>SDM+ + DS</td>
<td>1.99</td>
<td>27</td>
<td>N.S.</td>
</tr>
<tr>
<td>ST-FeSV</td>
<td>2.51</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

1. Twelve wells infected with 30 FFU for each chemical treatment. SDM+ (10 μg/ml) and/or DS (0.01 μg/ml) added 2 hr. p.i. as described in Materials and Methods.
2. (p) = Student's "t" test.
MAMA mixed with 0.01 µg/ml DS reduced the transformation frequencies by 41% (p=0.050) and 37% (p=0.010), respectively (Table 3).

**TABLE 3**

FAILURE OF DS TO ABROGATE THE INHIBITORY EFFECT OF MAMA

<table>
<thead>
<tr>
<th>Chemical Treatment</th>
<th>FFU/ml X 10^-1</th>
<th>% Reduction in FFU</th>
<th>(p) ²</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAMA</td>
<td>1.10</td>
<td>41</td>
<td>0.050</td>
</tr>
<tr>
<td>MAMA + DS</td>
<td>1.19</td>
<td>37</td>
<td>0.010</td>
</tr>
<tr>
<td>ST-FSfV</td>
<td>1.86</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1. Cells treated as described in Table 2. MAMA-1 ppm, DS-0.01 µg/ml.
2. (p) = Student's "t" test.

**DISCUSSION**

Cytotoxicity studies of water soluble fractions of SD or PD-derived JP5 fuels and DEM showed no differences in cellular toxicity. Effect on ST-FeSV transformation by SD or PD-derived JP5 were negative. In vivo animal studies reported by Rowland et al. (5) with 4 fractions from shale oil extracted in benzene reported only shale oil coke extract was carcinogenic and contained high concentrations of BaP. Major water soluble fractions of JP5 fuel contain no BaP, but do contain several aromatic components, none of which have been assayed in this system (6).

Methylazoxymethanol (MAM) is believed to be the proximate carcinogen of SDM and the ultimate carcinogen, a methyl diazomethane ion formed when the MAM undergoes oxidative metabolism (7). Thus, the biological specificity of this assay is suggested by the data shown in Tables 2 and 3. The inhibitory effect of SDM on virus transformation was abrogated by co-treatment with DS (Table 2), whereas the inhibitory effect of MAMA on virus transformation was not abrogated by DS (Table 3). Because DS inhibits biotransformation of SDM at the azomethane to azoxymethane step, co-treatment of DS and MAMA should not abrogate inhibition of virus transformation (7).

Contrasting results have been reported on the interaction of chemical carcinogens and retroviruses. For example,
vivo studies showed either an inhibitory (8,9), co-carcinogenic (10,11), or no effect (12,13) on transformation, depending upon the virus or chemical used in the experiments. In vitro studies with rat or mouse cells and viruses showed synergism (14-16).

Thus, HSF and ST-FeSV system show a time-dependent and, in most cases, a dose-dependent relationship in which diverse classes of chemicals (Table 4) inhibited ST-FeSV transformation when virus-infected cells were treated with non-toxic doses of chemicals at 2-6 hrs. p.i. Since the inhibitory effect was abrogated when cells were exposed 24 hrs. p.i., the temporal relationship between infection and treatment suggest chemical interference with either FeSV proviral synthesis or integration into host cell DNA.
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### TABLE 4
**CHEMICALS TESTED IN VIRUS INHIBITION ASSAY.**

<table>
<thead>
<tr>
<th>Chemical Group</th>
<th>Carcinogenic Activity</th>
<th>Inhibition of Virus Transformation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>I. Aromatic Amines</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. Naphthyl Amines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Two (2)</td>
<td>(+)</td>
<td>+</td>
</tr>
<tr>
<td>2. Phenyl-alpha</td>
<td>(+)</td>
<td>+</td>
</tr>
<tr>
<td>3. Phenyl-beta</td>
<td>(+)</td>
<td>+</td>
</tr>
<tr>
<td>B. N-Acetoxy 2 fluorenyleacetonamide</td>
<td>(+)</td>
<td>+</td>
</tr>
<tr>
<td><strong>II. Polycyclic Hydrocarbons</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Benzo(a)pyrene</td>
<td>(+)</td>
<td>+</td>
</tr>
<tr>
<td>2. Pyrene</td>
<td>(-)</td>
<td>-</td>
</tr>
<tr>
<td><strong>III. Hydrazines</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Hydrazine</td>
<td>(+)</td>
<td>+</td>
</tr>
<tr>
<td>2. Mono-methyl hydrazine</td>
<td>(+)</td>
<td>+</td>
</tr>
<tr>
<td>3. 1,1 dimethyl hydrazine</td>
<td>(+)</td>
<td>+</td>
</tr>
<tr>
<td>4. 1,2 dimethyl hydrazine</td>
<td>(+)</td>
<td>+</td>
</tr>
<tr>
<td><strong>IV. Other</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Aflatoxin B,</td>
<td>(+)</td>
<td>+</td>
</tr>
<tr>
<td>2. Asbestos</td>
<td>(+)</td>
<td>+</td>
</tr>
<tr>
<td>3. JP5 (SD)</td>
<td>(-)</td>
<td>-</td>
</tr>
<tr>
<td>4. JP5 (PD)</td>
<td>(-)</td>
<td>-</td>
</tr>
<tr>
<td>5. PJ5 (TH dimer)</td>
<td>(-)</td>
<td>-</td>
</tr>
<tr>
<td>6. Diesel fuel, Marine</td>
<td>(-)</td>
<td>-</td>
</tr>
<tr>
<td>7. Acetone</td>
<td>(-)</td>
<td>-</td>
</tr>
<tr>
<td>8. Methyl Azoxyethanolacetate</td>
<td>(+)</td>
<td></td>
</tr>
<tr>
<td>9. Formalin</td>
<td>(+)</td>
<td>+</td>
</tr>
<tr>
<td>10. Triton X-100</td>
<td>(?)</td>
<td></td>
</tr>
</tbody>
</table>

1. (+) considered a carcinogen, (-) non-carcinogen, from
(3) Unpublished data from Toxicology Branch, AMRL, WPAFB.
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ACKNOWLEDGMENTS

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REFERENCES


ENHANCED PERMISSIVENESS OF HUMAN CELLS TO FELINE RETROVIRUS INFECTION FOLLOWING HORMONE TREATMENT

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We previously reported human neonatal skin fibroblast cells treated with non-toxic concentrations of benzo (a) pyrene or aflatoxin B, inhibited feline sarcoma virus (FeSV) transformation 1. Although comparable levels of reverse transcriptase (RT) activity were found in both chemically treated and untreated cultures, infectious virus was not recoverable from either.

Others have documented the stimulation and activation of murine and primate retroviruses and enhanced FeSV transformation of human cells by optimal concentrations of glucocorticoid hormones 2-4.

The objectives of this study were to determine: 1) whether dexamethasone (D XM) induced both FeSV and FeLV synthesis in the apparent non-producer human cells and 2) whether feline oncornavirus-associated cell membrane antigen (FOCA) and group-specific antigen (GSA) were synthesized in addition to RT, in non-DXM treated cells.

Snyder-Theilen strain FeSV infected Detroit 550 neonatal human skin fibroblast cells produced very low levels of transforming virus when supernatant fluids from these cultures were titrated in normal D550 cells. The addition of 1.0 μg/ml DXM (2α-Fluoro-16α-methyl prednisolone) 24 hours post infection, significantly increased both FeSV and FeLV synthesis when compared to non-DXM treated infected cells (Table 1). The increase in titer ranged from a 56-fold increase to a 100-fold increase for FeSV, while FeLV increased from 0 in untreated cultures to 6 X 104 PFU/ml in DXM treated cultures, a 2.6 fold excess when compared to FeSV.
Table 1

DXM enhanced ST-FeSV and FeLV synthesis in D550 cells

<table>
<thead>
<tr>
<th>Virus and and</th>
<th>Virus Titer</th>
<th>Fold Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>( )</td>
<td>(-) DXM</td>
<td>(+) DXM</td>
</tr>
<tr>
<td>(1)</td>
<td>2.3</td>
<td>201.2</td>
</tr>
<tr>
<td>(2)</td>
<td>2.3</td>
<td>233.0</td>
</tr>
<tr>
<td>(3)</td>
<td>3.4</td>
<td>191.2</td>
</tr>
<tr>
<td>Untreated Control</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mean</td>
<td>2.7 ± 5</td>
<td>209.2 ±17.6</td>
</tr>
<tr>
<td>FeLV³</td>
<td>0</td>
<td>600.00</td>
</tr>
</tbody>
</table>

¹Determined by Students "t" test.

RT activity (cpm of §H dTMP incorporated/reaction/hr) ³ likewise increased. The values ranged from a 9-fold increase in 1 experiment to a 11-fold increase in 2 other separate experiments.

GSA⁶ and POCMA⁷ were detected in both FeSV infected DXM treated cells and FeSV infected cells only. Uninfected-cells, cell treated with 0.2 % acetone, and/or DXM were negative (Table 2).

Table 2

POCMA and GSA antigen expression in ST FeSV infected cells

<table>
<thead>
<tr>
<th>Cells Treated with¹</th>
<th>GSA</th>
<th>POCMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>FeSV</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>FeSV + DXM</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DXM</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FeSV + Acetone²</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Acetone</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>EMEM medium</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

¹Cells examined 72 hours post infection.
²0.2 % v/v acetone in complete EMEM medium.

Wu et al.⁸ described retrovirus infected cells as falling into 3 phenotypic categories with respect to virus gene expression: (1) virus producer cells; (2) non-producer cells with partial gene expression (any virus, component); and (3) cells not having any detectable viral gene products. In this study, D550 cells infected
with CT-FeSV could be classified as nonproducers with partial gene expression, i.e., transformation, reverse transcriptase, GSA and F0CHA antigens with little infectious virus being released. However, following DEM treatment, complete viral gene expression resulted with infectious (transforming) virus released.

The fact that DEM permits complete oncogenic viral gene expression in human cells is significant since the phenotypic expression of most human transformed cells has formerly been shown to be category 2 (non-producer) with partial gene expression.

ACKNOWLEDGEMENTS

Supported in part by a contract from AFSR, F49620-77-C-011 and the Graduate School, The Ohio State University.
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Paper Reprinted from Conference Proceedings No. 309

TOXIC HAZARDS IN AVIATION
Inhibition of Virus Transformation by High Energy Fuels as a Correlate of Carcinogenic Potential

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Summary

Hydrazine and naphthylamines and their derivatives were assayed for co-carcinogenic effects on ST FeSV-directed transformation of human cells. All chemicals tested at non-toxic concentrations showed anti-carcinogenic activity. The temporal relationship of chemical treatment to virus infection was more critical with the hydrazines than with the naphthylamines in that maximum anti-carcinogenic effect occurred when virus-infected cells were exposed to the hydrazines 2 hrs. post-infection, whereas the naphthylamines anti-carcinogenic effect was observed if cells were exposed either pre- or post-infection. The anti-carcinogenic effect, when compared with in vitro chemical transformation and neoplastic transformation, show a high degree of correlation. These data suggest this assay system may lend itself to a rapid screen (9-13 days) of chemicals for carcinogenic potential. Cytotoxic results showed no significant difference in shale oil or petroleum derived 3P3 or DFM. Co-carcinogenic potential of 3P3, 3P10, R15, and DFM are being evaluated.

Introduction

Previous studies from this laboratory showed chemical carcinogens inhibited virus-directed feline sarcoma virus transformation of human skin fibroblast (HSF) cells at non-toxic concentrations (1). The carcinogen treatment inhibited a specific virus gene function, i.e., transformation, but not virus synthesis. Other viral gene products, RNA-dependent DNA polymerase (RDDP), Group-Specific Antigens (GSA) and Feline Oncornavirus Associated Cell Membrane Antigen (FOCAM), were detected in both carcinogen-treated or non-treated virus infected cells (2). These studies suggested the inhibitory effect on virus-directed transformation was mediated by the carcinogen, while the inhibitory effect on complete virus synthesis was cell mediated. Further, the inhibitory effect of the carcinogens was abrogated when chemicals were added to virus-infected cells 48 hrs. post-infection.

Hydrazine and its derivatives have widespread use in medicine, agriculture, and aerospace research (3). While many biologic effects of hydrazine (H2 and its derivatives, monomethyl hydrazine (MMH), 1,1 dimethylhydrazine (UDMH), and 1,2 dimethylhydrazine (SDMH) have been studied in animals, extrapolation of these biologic effects to man has been difficult because of differential responses manifested in diverse species of test animals. Additionally, the different chemical properties of each of these chemicals has led to differential physiological responses within the same species. For example, Diwan et al. (4) concluded that genetic differences with inbred strains of mice affected the response of DMH carcinogenesis. Thus, carcinogenesis assays in rodents may lead to false negative results based on that genetic strain used in the assay. Ideally, economic, short-term, reliable in vitro assays would be invaluable in determining carcinogenic potential of chemicals.

In this study, we examined the co-carcinogenic effect of alpha-naphthylamine (ANA), phenyl-alpha naphthylamine (PANA), phenyl-beta naphthylamine (PBNA), H2, MMH, UDMH, and SDMH on Snyder-Theilen feline sarcoma virus (ST-FeSV) transformation of HSF. The effects were further correlated with in vitro chemical transformation of HSF described by Milo and Blakeslee (3). Cytotoxic analyses of petroleum and shale oil derived jet fuels are also presented.

Materials and Methods

Cells

Human foreskin fibroblast cells (Detroit 530-CCL109, American Type Culture Collection, Rockville, MD) were grown in Minimal Essential Medium with Earles salts supplemented with 1.0 mM sodium pyruvate, 2 mM glutamine, 1% non-essential amino acids, 50 μg/ml gentamycin (Schering Diagnostics, Port Reading, NJ) and 10% fetal bovine serum (Sterile Systems, Logan, UT) thereafter designated Complete Medium (CM). Cells were serially passaged every 3-4 days at 1:2 split ratios and incubated at 37°C in 5% CO2.

Virus

The preparation of stock ST-FeSV was described previously (6). Briefly, 10% cell-free homogenates were prepared and frozen at -85°C in L15 medium and 5% FBS.

Co-Chemical Virus Assays

Preconfluent log phase growth HSF cells were trypsinized and 4 X 10⁶ cells seeded onto 16 mm wells (Costar, Cambridge, MA) in 1.0 ml CM and incubated 18 hrs. prior to treatment. Cells pre-treated with chemicals prior to virus infection were incubated 90 min. with appropriate chemical concentration at 2, 6, or 24 hr. pre-infection. Cells were washed 2X in CM, refed and incubated at 37°C until virus infection. Cells to be infected were washed with serum-free CM and treated with 25 units/ml DEAE-Dextran (Sigma, St. Louis, MO) in serum-free CM. After 20 min., the cells were washed with CM + 5% FBS, infected with 0.05 ml ST-FeSV, diluted to 1,000 focus forming units (FFU) per ml. Twelve wells were used for each time period. Plates were rocked at 10-15 min. intervals and virus adsorbed 2 hr. at 37°C. After adsorption, the inocula were aspirated and cells refed with 2.0 ml CM. Virus infected cells were treated at 2, 6, or 24 hr. following virus adsorption by incubating infected cells with designated concentration of chemical for 90 min. followed by washing and refeding cells with 2.0 ml CM. The cells were refed with CM on the 6th day post-infection (PI), and subsequently fixed with 10% phosphate buffered formalin and stained with Giemsa 3-4 days later. Foci were counted at 25-40X with a dissecting microscope in non-treated (control) and chemically treated wells. FFU ± S.D. were determined for each treatment time and significance determined by Student's 't' test.
Chemicals

ANA, PANA, and PBNA were dissolved in spectral grade acetone at 1.0 mg/ml. Prior to use, dilutions were made in CM to experimental concentrations. HZ, MMH, and UDMH were pipetted into CM at 1 X 10^6 ppm and diluted in CM to experimental concentrations. SDMH was dissolved in 0.1 N HCl at a concentration of 10 mg/ml and diluted in CM to experimental concentrations.

Both petroleum or shale oil derived fuels were dispersed in equal volumes of 20% Triton-X (Sigma, St. Louis, MO) serum-free CM with shaking. Dilutions tested were from 5000 to 0.3 ppm. Eight wells, seeded with 500 cells/well, were treated for 90 min., washed and refed with CM supplemented with 20% FBS. Cultures were incubated 9-10 days, fixed in 10% buffered formalin, stained with Giemsa, and clones containing at least 50 cells were counted. Toxicity was determined by dividing the average number of clones in treated wells by the average number of clones in control wells. LD_{50} values were determined by plotting percent survival (ordinate) against concentration (Abscissa, log scale) on 3-cyclic semi-logarithmic graph paper. LD_{50}'s were then determined by inspection.

Results

Naphthylamines

PANA treatment resulted in a dose-related suppression of transformation (Fig. 1A). Cells treated with 10 µg/ml resulted in significant inhibition of transformation at all time periods tested. Results with 20 µg/ml were similar, although treatment at 6 hrs. post-infection approached control values. Cells treated with 0.1 µg/ml showed no difference in transformation frequencies from untreated controls.

Treatment with 10 µg/ml PBNA resulted in suppression at all time periods tested (Fig. 1B). Inhibition of transformation ranged from values of 40% to 63% inhibition. However, cells exposed to 0.01 µg/ml before ST-FeSV infection resulted in enhanced focus formation at 2 hrs., whereas significant inhibition was observed when virus infected cells were treated 6 hrs. post-infection.

Figure 1C shows the effect of 10 µg and 0.01 µg/ml ANA. Both concentrations used inhibited ST-FeSV transformation, with the most significant inhibition observed when cells were treated from 2-6 hrs. pre-infection. Cells treated 6 hrs. or 24 hrs. post-virus infection had no effect on virus transformation.

Hydrazines

MMH (100 ppm and 10 ppm) significantly enhanced virus transformation in a dose-dependent manner when cells were exposed 2 hrs. pre-infection (Fig. 2A). Conversely, treatment of virus-infected cells resulted in significant inhibition of transformation when cells were treated 2 hrs. or 6 hrs. post-infection. No effect was noted at 24 hrs. post-infection.

SDMH (100 µg/ml and 10 µg/ml) likewise enhanced virus transformation when cells were treated 2 hrs. pre-infection (Fig. 2B). In virus infected cells, SDMH treatment inhibited transformation by values ranging from 20% (10 µg/ml) to 30% (100 µg/ml). The inhibitory effect was observed at 2 hrs. post-infection, but not at 6 or 24 hrs. post-infection.

The effects of UDMH on virus transformation are shown in Fig. 2C. One hundred or 10 ppm exposure to UDMH inhibited transformation at 3 different time periods in relation to virus infection. UDMH inhibited transformation by values ranging from 25% to 50% when cells were exposed 6 hrs. before or 2 hrs. and 24 hrs. post-infection.

The results of HZ (60 ppm and 6 ppm) exposure are shown in Fig. 2D. Fig. 2D is a composite figure of 3 separate experiments showing HZ effects on virus transformation. Like SDMH and MMH, cells exposed to HZ 2 hrs. pre-infection, significantly enhanced virus transformation whereas exposure 2 hrs. post-virus infection significantly inhibited virus transformation as did MMH, SDMH, and UDMH.

Cytotoxicity of Fuels

Petroleum derived (PD) or shale oil derived (SOD) JP5 or diesel fuel, marine (DFM) cytotoxic analyses are shown in Table 1. The results show no significant difference in toxicity values between SOD or PD fuels. LD_{50}'s for SOD, JP5 and PD-JP5 were 102 ppm and 100 ppm, respectively, SOD-DFM and PD-DFM were 85 ppm and 87 ppm. LD_{50}'s for JP10 and RJS were 91 ppm and 19 ppm, respectively.

<table>
<thead>
<tr>
<th>Fuel</th>
<th>Derived From</th>
<th>LD_{50} (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>JP5</td>
<td>Shale</td>
<td>102</td>
</tr>
<tr>
<td>JP5</td>
<td>Petroleum</td>
<td>100</td>
</tr>
<tr>
<td>DFM</td>
<td>Shale</td>
<td>85</td>
</tr>
<tr>
<td>DFM</td>
<td>Petroleum</td>
<td>87</td>
</tr>
<tr>
<td>RJS</td>
<td>Petroleum</td>
<td>19</td>
</tr>
<tr>
<td>JP10</td>
<td>Petroleum</td>
<td>91</td>
</tr>
</tbody>
</table>

1 DFM = diesel fuel, marine
Fig. 1. HSF cells were plated in 16 mm diameter wells with 2.0 ml CM and incubated overnight. Cells were treated with ANA as described in Materials and Methods. (-) indicates cells treated before virus infection (hrs). (+) indicates cells treated after virus infection (hrs). * = significance determined by Student t test. A-PANA, B-PBNA and C-ANA.
Fig. 2. HSF cells were plated in 16 mm diameter wells with 2.0 ml CM and incubated overnight. Cells were treated with ANA as described in Materials and Methods. (+) indicates cells treated before virus infection (hrs). (-) indicates cells treated after virus infection (hrs). * - significance determined by Student "t" test. A-MMH, B-SDMH, C-UDMH and D-Hz. D is a composite of 3 separate tests. Each point - n = 48 ± S.D.
Discussion

The co-carcinogenic effects of hydrazine and its derivatives and naphthylamines described in this report, when correlated with in vitro chemical transformation and neoplastic transformation, show a high degree of correlation (Table 2). ANA, PANA, and PBNA showed 100% correlation with the two parameters, whereas MMH and SDMH showed activity in the co-carcinogenesis assays, but not in the carcinogenesis assays. HZ and UDMH, like the naphthylamines, showed 100% correlation with in vitro chemical transformation and neoplastic transformation. The inhibition of transformation from chemical treatment was not a result of cell killing in that sub-toxic concentrations were used.

Table 2. Correlation between inhibition of virus transformation, in vitro chemical transformation and neoplastic transformation.

<table>
<thead>
<tr>
<th>Chemical Group</th>
<th>In Vitro Chemical Transformation</th>
<th>Neoplastic Transformation</th>
<th>Inhibition of ST-FeSV Transformation</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Naphthylamines</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. ANA</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>2. PANA</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>3. PBNA</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>II. Polycyclic Hydrocarbons</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. BAP²</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>2. Pyrene</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>III. Hydrazines</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. HZ</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes³</td>
</tr>
<tr>
<td>2. MMH</td>
<td>No</td>
<td>No</td>
<td>Yes³</td>
</tr>
<tr>
<td>3. UDMH</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes³</td>
</tr>
<tr>
<td>4. SDMH</td>
<td>No</td>
<td>No</td>
<td>Yes³</td>
</tr>
</tbody>
</table>

1 Courtesy Dr. George Milo
2 BAP - Benzo(a)pyrene
3 Significant enhancement - 2 hrs. pre-infection

Enhanced virus transformation by HZ, MMH, and SDMH observed when cells were exposed 2 hrs. pre-infection, may be related to cell growth stimulation shown by these chemicals in dose survival studies (data not shown). Similar findings of cell stimulation have been observed with murine and feline lymphocyte cultures (7). The major effect on virus transformation (inhibition) occurred with all test chemicals when virus infected cells were exposed to the appropriate concentrations. The temporal relationship of chemical treatment to virus infection appears more critical with the naphthylamines than with the hydrazines and that maxium inhibition occurred when virus infected cells were exposed to the hydrazines 2 hrs. post-infection, whereas this inhibitory effect was observed at all 6 time periods with PANA and PBNA and at 3 time periods with ANA.

Cytotoxicity results showed no significant difference in shale oil or petroleum derived fuels. Co-carcinogenic effects with ST-FeSV are being evaluated. HZ, MMH, UDMH, SDMH, and PANA have shown mutagenic, teratogenic, or carcinogenic properties depending upon the assay used (7-11). Thus, these chemicals interact with host cell transcription or translational processes. In previous studies, we concluded the inhibitory (anti-carcinogenic) effect of benzo(a)pyrene, aflatoxin B1 or N-acetoxy-2 fluorenyl acetamide on virus transformation was not due to decreased cellular proliferation or virus synthesis (1,2). Further, the anti-carcinogenic effect was abrogated when cells were exposed >24 hrs. post-infection. The temporal relationship between infection and treatment suggested chemical interference with FeSV proviral synthesis or integration into host cell DNA.

Contrasting results have been reported on the interaction of chemical carcinogens and oncogenic RNA viruses. For example, in vivo studies showed either an anti-carcinogenic (12,13), co-carcinogenic (14,15) or no effect (16,17) on transformation depending on the virus or chemical used in the experiments. Whereas in vitro studies with rat or mouse cells showed synergism (18-20).

We previously reported anti-carcinogenic activity with three known carcinogens on FeSV transformation of human cells in vitro. A recent report by Rhim and Arnstein (21) described anti-carcinogenic activity of an oncogenic murine virus on chemical-induced transformation of canine cells.

Thus, the mechanism(s) of chemical, virus, or co-chemical-virus transformation remain unknown and further studies are warranted to evaluate these interactions.
References


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