INFORMATION TO USERS

While the most advanced technology has been used to photograph and reproduce this manuscript, the quality of the reproduction is heavily dependent upon the quality of the material submitted. For example:

- Manuscript pages may have indistinct print. In such cases, the best available copy has been filmed.
- Manuscripts may not always be complete. In such cases, a note will indicate that it is not possible to obtain missing pages.
- Copyrighted material may have been removed from the manuscript. In such cases, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, and charts) are photographed by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each oversize page is also filmed as one exposure and is available, for an additional charge, as a standard 35mm slide or as a 17”x 23” black and white photographic print.

Most photographs reproduce acceptably on positive microfilm or microfiche but lack the clarity on xerographic copies made from the microfilm. For an additional charge, 35mm slides of 6”x 9” black and white photographic prints are available for any photographs or illustrations that cannot be reproduced satisfactorily by xerography.
Mouse skin tumor initiation by ionizing radiation and the detection of dominant transforming gene(s)

Jaffe, Deborah Ruth, Ph.D.
The University of Arizona, 1987
PLEASE NOTE:

In all cases this material has been filmed in the best possible way from the available copy. Problems encountered with this document have been identified here with a check mark ✓.

1. Glossy photographs or pages ✓
2. Colored illustrations, paper or print
3. Photographs with dark background ✓
4. Illustrations are poor copy
5. Pages with black marks, not original copy ✓
6. Print shows through as there is text on both sides of page
7. Indistinct, broken or small print on several pages
8. Print exceeds margin requirements
9. Tightly bound copy with print lost in spine
10. Computer printout pages with indistinct print
11. Page(s) lacking when material received, and not available from school or author.
12. Page(s) seem to be missing in numbering only as text follows.
13. Two pages numbered. Text follows.
14. Curling and wrinkled pages ✓
15. Dissertation contains pages with print at a slant, filmed as received
16. Other

_________________________________________________________
_________________________________________________________

University Microfilms International
MOUSE SKIN TUMOR INITIATION BY IONIZING RADIATION
AND THE DETECTION OF DOMINANT TRANSFORMING GENE(S)

by
Deborah Ruth Jaffe

A Dissertation Submitted to the Faculty of the
DEPARTMENT OF MOLECULAR AND CELLULAR BIOLOGY
In Partial Fulfillment of the Requirements
For the Degree of
DOCTOR OF PHILOSOPHY
WITH A MAJOR IN MOLECULAR BIOLOGY
In the Graduate College
THE UNIVERSITY OF ARIZONA

1987
As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Deborah Ruth Jaffe entitled Mouse Skin Tumor Initiation by Ionizing Radiation and the Detection of Dominant Transforming Gene(s) and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Dissertation Director Date
STATEMENT BY AUTHOR

This dissertation has been submitted in partial fulfillment of requirements for an advanced degree at The University of Arizona and is deposited in the University Library to be made available to borrowers under rules of the Library.

Brief quotations from this thesis are allowable without special permission, provided that accurate acknowledgement of source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the major department or the Dean of the Graduate College when in his or her judgment the proposed use of the material is in the interests of scholarship. In all other instances, however, permission must be obtained from the author.

SIGNED: [Signature]
ACKNOWLEDGEMENTS

I wish to thank the members of my committee; Drs. G. Tim Bowden, Eugene W. Gerner, John J. Duffy, A. Jay Gandolfi and I. Glenn Sipes for all their help.

I am indebted to Dr. J.N. Shively for histopathological evaluations and Dr. D. Sim for statistical analysis. I would also like to thank Drs. J. Williamson and W. Lutz for calculating the dosimetry and designing the irradiation box.

Special thanks to my family for their support and encouragement throughout my graduate studies.

I am indebted to my friends and colleagues from the Department of Radiation Oncology who helped to make science fun and interesting. I am especially grateful to Joanne Finch for her endless ability to listen and her eternal optimism.

Special thanks to Kevin Lewis for his encouragement, constructive and challenging discussions and most important, his friendship.

Finally, an appreciative note to Sally Anderson for her tireless efforts in typing this manuscript.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>LIST OF ILLUSTRATIONS</th>
<th>vi</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF TABLES</td>
<td>vii</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>ix</td>
</tr>
<tr>
<td>1. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>Ionizing Radiation Evidence for Carcinogenic Properties in Humans</td>
<td>2</td>
</tr>
<tr>
<td>Radiation Effects on Skin</td>
<td>7</td>
</tr>
<tr>
<td>Multi-Stage Mouse Skin Model</td>
<td>9</td>
</tr>
<tr>
<td>Ionizing Radiation - Experimental Evidence Related to Skin Tumor Induction in Rodents</td>
<td>13</td>
</tr>
<tr>
<td>Oncogenes: History</td>
<td>15</td>
</tr>
<tr>
<td>DNA Transfection</td>
<td>19</td>
</tr>
<tr>
<td>Functional Role of Proto-Oncogenes</td>
<td>21</td>
</tr>
<tr>
<td>Mechanisms of Activation</td>
<td>24</td>
</tr>
<tr>
<td>Role of Oncogenes in Carcinogenesis</td>
<td>28</td>
</tr>
<tr>
<td>2. IONIZING RADIATION AS AN INITIATOR IN THE MOUSE TWO-STAGE MODEL OF SKIN TUMOR FORMATION</td>
<td>36</td>
</tr>
<tr>
<td>Introduction</td>
<td>36</td>
</tr>
<tr>
<td>Materials and methods</td>
<td>38</td>
</tr>
<tr>
<td>Results</td>
<td>41</td>
</tr>
<tr>
<td>Discussion</td>
<td>50</td>
</tr>
<tr>
<td>3. IONIZING RADIATION AS AN INITIATOR: EFFECTS OF PROLIFERATION AND PROMOTION TIME ON TUMOR INCIDENCE</td>
<td>55</td>
</tr>
<tr>
<td>Introduction</td>
<td>55</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>57</td>
</tr>
<tr>
<td>Results</td>
<td>59</td>
</tr>
<tr>
<td>Discussion</td>
<td>67</td>
</tr>
</tbody>
</table>
TABLE OF CONTENTS--Continued

<table>
<thead>
<tr>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4. IONIZING RADIATION ENHANCES MALIGNANT PROGRESSION OF MOUSE SKIN TUMORS............................ 75</td>
</tr>
<tr>
<td>5. DETECTION AND CHARACTERIZATION OF DOMINANT TRANSFORMING GENES IN RADIATION-INDUCED MOUSE SKIN TUMORS...................... 84</td>
</tr>
<tr>
<td>Introduction........................................... 84</td>
</tr>
<tr>
<td>Materials and Methods..................................... 86</td>
</tr>
<tr>
<td>Nucleic Acid Extraction.................................. 86</td>
</tr>
<tr>
<td>Transfection Assays....................................... 88</td>
</tr>
<tr>
<td>Restriction Endonuclease Functional Mapping.................... 89</td>
</tr>
<tr>
<td>Growth in Soft Agar...................................... 89</td>
</tr>
<tr>
<td>Tumorigenicity in Nude Mice................................ 90</td>
</tr>
<tr>
<td>Southern Analysis.......................................... 90</td>
</tr>
<tr>
<td>Northern Analysis.......................................... 91</td>
</tr>
<tr>
<td>Probes.......................................................... 91</td>
</tr>
<tr>
<td>Results....................................................... 92</td>
</tr>
<tr>
<td>Detection of Dominant Transforming Genes......................... 92</td>
</tr>
<tr>
<td>Probing for Activated Ras Genes............................ 94</td>
</tr>
<tr>
<td>Characterization of the Transformed Phenotype................... 95</td>
</tr>
<tr>
<td>Screening Transfectants for the Presence of Known Cellular Oncogenes........................................ 99</td>
</tr>
<tr>
<td>Expression of c-myc in Mouse Skin Tumors...................... 104</td>
</tr>
<tr>
<td>Amplification and Rearrangement of c-myc in Mouse Skin Tumors............................................... 106</td>
</tr>
<tr>
<td>Discussion.................................................... 110</td>
</tr>
<tr>
<td>6. CONCLUSIONS............................................... 120</td>
</tr>
<tr>
<td>REFERENCES .................................................. 129</td>
</tr>
</tbody>
</table>
# LIST OF ILLUSTRATIONS

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Schematic of the Irradiation Protocol</td>
<td>40</td>
</tr>
<tr>
<td>2.</td>
<td>Time to First Tumor</td>
<td>48</td>
</tr>
<tr>
<td>3.</td>
<td>The Effect of TPA Pretreatment on Time to First Tumor Taking Into Account Only the Number of Animals at Risk</td>
<td>49</td>
</tr>
<tr>
<td>4.</td>
<td>Time Dependent Tumor Incidence in CD-1 Mice After a Subcarcinogenic Dose of Radiation</td>
<td>61</td>
</tr>
<tr>
<td>5.</td>
<td>Effect of TPA Pretreatment on Time Dependent Tumor Incidence in CD-1 Mice After a Subcarcinogenic Dose of X-rays</td>
<td>65</td>
</tr>
<tr>
<td>6.</td>
<td>Schematic of Irradiation Protocol</td>
<td>79</td>
</tr>
<tr>
<td>7.</td>
<td>Time Dependent Tumor Incidence and Rate of Malignant Conversion in CD-1 Animals</td>
<td>81</td>
</tr>
<tr>
<td>8.</td>
<td>Southern Blot Analysis of Primary NIH3T3 (8A) and Secondary Rat-2 (8B) Transfectants Hybridized to Ha-, Ki- or N-ras Probes</td>
<td>96</td>
</tr>
<tr>
<td>9.</td>
<td>Southern Blot Analysis of Transfectant DNAs Digested with XbaI and Hybridized to a Viral erbB Probe</td>
<td>101</td>
</tr>
<tr>
<td>10.</td>
<td>Southern Blot Analysis of Primary and Secondary NIH3T3 Transfectant DNAs Hybridized with BamHI/EcoRI and a Human neu Gene (psv2-neu)</td>
<td>102</td>
</tr>
<tr>
<td>11.</td>
<td>Southern Blot Analysis of NIH3T3 Transfectant DNAs Digested with PstI and Hybridized to a 1.6 Kbp EcoRI/HindIII Insert of Human c-raf-1</td>
<td>103</td>
</tr>
</tbody>
</table>
LIST OF ILLUSTRATIONS--Continued

Figure

12. Northern Blot Analysis of Total RNAs Hybridized to a 1.5 Kb PstI Insert of Viral myc (pmyc-2).................105
13. Northern Blot Analysis of Tumor RNAs Hybridized to a 600 bp EcoRI/BamHI Insert of Mouse Ha-ras (psp659EB2-ras).....107
14. Northern Dot Blot Analysis of Total Tumor and Tissue RNA..................................................108
15. Southern Blot Analysis of Tumor DNAs Digested to Completion with BamHI and Hybridized to a 1.5 Kb PstI Insert of Viral myc (pmyc-2)..............................109
16. Structure and Restriction Map of the Mouse and Human c-myc Genes........................................117
LIST OF TABLES

Table                        Page

1. Relative Sensitivities of Various Human and RFM Mice Tissues to the Induction of Radiogenic Cancer.................. 6
2. Proto-oncogene Activation in Animal Tumor Models.............. 31
3. Tumor Incidence in Sencar Mice Initiated with Ionizing Radiation and Promoted with TPA................................. 42
4. Effects of TPA Pretreatment on Tumor Incidence in Sencar Mice Initiated with Ionizing Radiation and Promoted with TPA........................................ 45
5. Incidence of Other Tumor Types in Sencar Mice Initiated with Ionizing Radiation............................................ 47
6. Tumor Incidence in CD-1 Mice Initiated with Ionizing Radiation and Promoted with TPA........................................ 62
7. Effect of TPA Pretreatment on Tumor Incidence..................... 66
8. Conversion of Papillomas to Squamous Cell Carcinoma.............. 68
9. Carcinoma Incidence in CD-1 Mice Initiated with Ionizing Radiation............................................................... 69
10. Transforming Efficiency of Radiation Induced Tumors.............. 93
11. Characterization of NIH3T3 Transfected Cell Lines in Terms of Anchorage Independent Growth and Tumorigenicity in Nude Mice............................................. 97
12. Restriction Endonuclease Functional Maps of NIH3T3 Transfected Cell Lines..................................................... 100
ABSTRACT

The initiating potential of a range of 4 MeV X-rays was studied using the mouse skin two-stage model of carcinogenesis. A single dose of radiation was followed by promotion with 12-O-tetradecanoyl phorbol-13-acetate (TPA). The effect of TPA on tumor incidence when applied as a single dose 24 hours prior to irradiation was examined. Studies were also designed to investigate the effect of promotion duration on tumor incidence. Animals were promoted with TPA for 10 or 60 weeks. Evidence presented here indicates that ionizing radiation can act as an initiator in this model system. All animals that were promoted with TPA for the same duration had a similar incidence of papillomas (pap) regardless of radiation or TPA pretreatment. However, squamous cell carcinomas (scc) arose only in animals that were initiated with ionizing radiation followed by TPA promotion. Increasing the promotion duration enhanced the incidence of scc at the lower initiation dose. TPA pretreatment at the higher irradiation dose resulted in an overall decrease in tumor incidence. At the lower dose of radiation, TPA pretreatment resulted in an increase in the incidence of scc. The incidence of basal cell carcinomas (bcc) was dose dependent and appeared to be independent of TPA promotion. Although ionizing radiation acts as a weak initiator in mouse skin, the conversion of pap to scc was higher than that reported for chemicals initiators. To test this further animals were initiated with
N-methyl-N' nitro-N-nitrosoguanidine (MNNG) followed by biweekly promotion with TPA. After 20 weeks of promotion, the animals were treated with either acetone, TPA or 8 fractions of 1 MeV electrons. Data indicate that the dose and fractionation protocol used in this study enhanced the progression of pre-existing pap.

To examine the role of oncogene activation in radiation induced mouse skin tumors, DNA from various tumors (pap, bcc, scc) were examined for the presence of dominant transforming activity by the NIH3T3 and Rat-2 focus assays. Dominant transforming activity was observed in all tumor types but not in normal or treated epidermis or corresponding liver. The transformed phenotype was further confirmed by growth in soft agar and tumorigenicity in Nude mice. Southern blot hybridization to ras (Ha, Ki, N), raf, neu, erbB and β-lym indicate that these genes are not responsible for the observed transforming activity. These data suggest that the the oncogenic sequences activated in these tumors are unique. The work presented here also provides evidence for novel c-myc transcripts and corresponding genomic rearrangements in a few of the tumors studied.
CHAPTER 1

INTRODUCTION

Ionizing radiation is a normal component of our environment. Low levels of background ionizing radiation originate from the earth and outer space. The use of ionizing radiation for diagnostic, therapeutic, and energy purposes, as well as the increase in nuclear accidents and weapons testing, has increased the concern over both short and long term biological effects. The late effect of ionizing radiation on animals and humans include increases in mutation rate resulting in genetic damage and the induction of cancer. The focus of this dissertation was the study of radiogenic-induced skin cancer.

Cancer induction is a multistage process; the initial stages may depend on specific lesions in DNA and their misrepair or lack of repair. This, in turn, may result in mutations and/or chromosomal rearrangements. The exact mechanisms involved in this process have not been determined. The interaction of the basic process of initiation with the subsequent processes of progression and tumor development has not been fully characterized.

The research presented in this dissertation has addressed some fundamental aspects of radiation carcinogenesis using the mouse skin multistage model. Data on the initiating potential of ionizing...
radiation is presented in Chapters 2 and 3. The effects of ionizing radiation on the conversion of benign skin papillomas to squamous cell carcinomas is discussed in Chapter 4. In Chapter 5, data are presented concerning the molecular biology of these radiation induced tumors. DNAs isolated from these tumors have been characterized in terms of transforming potential and restriction endonuclease functional maps have been prepared. The potential role of some known cellular proto-oncogenes was also examined. Finally, the conclusion summarizes and reviews the data in terms of currently postulated mechanisms for the carcinogenic effects of radiation.

Ionizing Radiation

Evidence for Carcinogenic Properties in Humans

Cancer induced by ionizing radiation is a disease new to human history. It did not become an important problem until opportunities for human exposure were increased by the discovery of x-rays in 1895 and radioactivity in 1896 (Stone-Scott, 1897). Radiation-induced cancer is primarily a disease of the 20th century, although the first significant exposures from non man-made sources occurred earlier than this. In 1400 A.D. uranium was being mined in central Europe. Although lung cancer had been documented in the miners, the etiological role of radon was not suspected until 1932. Even though mortality was high among the miners, the number at risk were only a few hundred at any one time and were localized in two small areas of central Europe. Such small foci of disease, however
lethal, are not comparable with the great epidemics and pandemics induced by enteric organisms in mankind's past. Except in the event of nuclear war or a major catastrophic nuclear accident, it seems unlikely that radiogenic cancer will have an impact on future society comparable to the many diseases of the past and a dreaded new sickness of the present and future, AIDS. Nevertheless, the hazard is real and and the human population continues to be at risk from exposure to ionizing radiation.

Analyses of the effects of ionizing radiation on man are based upon: 1) occupational exposures; 2) therapeutic and diagnostic exposures; 3) accidental exposures; and 4) atomic bomb exposure. For a number of human malignancies there is very strong epidemiological evidence identifying radiation as a causative agent. Epidemiologic studies of populations exposed to radiation have shown that a wide range of cancers can be induced by radiation (Schull, 1984). Medical practices have also demonstrated a clear association between irradiation and subsequent development of cancer (Scanlan, Berk and Khandekart, 1979).

Perhaps the most extensive data on the carcinogenic effects of ionizing radiation comes from detailed follow-ups of the approximately 109,000 survivors and controls of the atomic bombs dropped on Nagasaki and Hiroshima in 1945 (Kohn and Fry, 1984). The average dose of radiation that these individuals received has been estimated to be 0.27 Gy and at present detailed analysis of data represents a follow-up period of over 30 years. A significant
increase in the incidence of leukemias was observed prior to any other malignancy. This malignancy has a relatively short latent period. Subsequently, cancers of almost all organs have been found to be increased significantly (Kohn and Fry, 1984).

There are reports in the literature as early as 1902 on the use of radiation in the treatment of benign diseases (Friedlander, 1970). Multiple benign conditions including enlarged thymus, tonsils and adenoids, tinea capitis and acne were ultimately treated in infancy, childhood and adolescence with external radiation (Scanlan et al., 1979; Shore, Woodward and Hempelam, 1984). Advances in the treatment of childhood and young adult cancers have significantly prolonged the survival of these individuals. Unfortunately, the occurrence of new tumors associated with the initial radiation therapy among these patients is becoming a major concern. The development of new lesions within a previously irradiated field after a 10-20 year latency period strongly implicates ionizing radiation as the principal etiological factor. Strong evidence is emerging which indicates that patients with a history of prior irradiation, for benign or malignant lesions, especially to the head and neck area, are at increased risk of developing subsequent neoplasms. Thus, there is little doubt of the carcinogenic potency of ionizing radiation in humans (Friedlander, 1970; Li, Cassady and Jaffe, 1975; Southwich, 1977; Scanlan et al., 1979; Valagussa et al., 1980; Boivin and Hutchinson, 1984; Sandove, 1984; Shore et al., 1984).
Ionizing radiation is a relatively weak carcinogen in man. Exposure to 0.01 Gy at a low dose rate carries a lifetime risk of 1 in 10,000 of developing a fatal radiation induced cancer. If it is assumed that 20 percent of all deaths in this country result from cancer, an exposure to 20 Gy would be required to double the risk of dying from cancer. This is $10^4$ times the annual dose received from natural background radiation (Upton, 1986). Although ionizing radiation can induce both benign and malignant tumors in any mammalian cell type, there is a wide range of tissue sensitivities and susceptibilities to cancer induction. Some of the differences in tissue sensitivities are shown for both humans and mice in Table 1. Every tumor type known to be induced by ionizing radiation also appears spontaneously in unirradiated individuals. Ionizing radiation has been shown to increase total tumor incidence, and in many cases, decrease the latency period of cancer development in man. The study of the biological and carcinogenic effects of radiation will continue to receive impetus from the expanding use of radiologic methods in medicine, science and industry. The recent nuclear accident at Chernobyl has added impetus for further scientific study of the long term effects of varying degrees of radiation exposure and the always elusive estimate of potential health risk to low dose exposure.
Table 1.
Relative Sensitivities of Various Human and RFM Mice Tissues to the Induction of Radiogenic Cancer *

<table>
<thead>
<tr>
<th>High Sensitivity</th>
<th>Moderate Sensitivity</th>
<th>Low Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>Myelopioetic tissue</td>
<td>Breast</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lung</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Salivary Gland</td>
</tr>
<tr>
<td>Mice</td>
<td>Thymus</td>
<td>Pituitary</td>
</tr>
<tr>
<td></td>
<td>Ovary</td>
<td>Uterus</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Breast</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Myelopioetic</td>
</tr>
<tr>
<td></td>
<td></td>
<td>tissue</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lung</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Haderian Gland</td>
</tr>
</tbody>
</table>

Radiation Effects on Skin

It is of interest to note that in both humans and mice, skin is relatively radioresistant when compared to other organs (Table 1). Results in the literature suggest that human and rat skin are equally susceptible to the carcinogenic action of x-rays under comparable conditions of exposure when allowance is made for the different times needed for expression of tumor development in the two species (Albert et al., 1976; Burns et al., 1976). Mouse skin, on the other hand, appears to be more radioresistant than either human or rat skin. The reason for this difference in radiosensitivities is not known.

The ability of ionizing radiation to function as a complete skin carcinogen has been recognized for almost a century. Less than a year after Roentgen's discovery in 1895, visible skin damage in humans was observed following x-ray exposure (Sanders and Kathren, 1983). Similar changes were also noted after the discovery of radium in 1898 (Potten, 1985). Cutaneous neoplasms that arose from these early exposures developed after long latent periods and were reported to be squamous cell carcinomas. The different cell types within the skin structure vary in their radiosensitivity. The intermitotic basal cells of the epidermis are among the most radiosensitive cells in the body, even though the skin itself is relatively radioresistant. In the dermis, the melanocytes and connective tissue are extremely radioresistant, while the follicular epithelium is as radiosensitive as the intermitotic basal cells of
the epidermis (Casavette, 1980).

The acute reaction of the skin in response to ionizing radiation results from damage to the germe cells of the epidermis and its appendages. Radiation induces mitotic delay in the basal cells, the duration of which is related to the cell's age in the cell cycle and the dose of ionizing radiation received. A decrease in the mitotic index, reproductive failure, abnormal mitosis and depletion of basal cells are all commonly observed short term skin responses to ionizing radiation. The maturation of cells originating in the basal layer and their transit time through the various epidermal cell layers is not altered in response to ionizing radiation. Recovery is manifested by accelerated reproduction rates in the clonogenic cells accompanied by an increase in the labeling index and a decrease in the cell cycle time (Potten, 1985). Long term effects, other than cancer, have been shown to be a result of damage to the vasculature. Changes in the endothelium of the microvasculature, swelling, degeneration, necrosis and inflammation all lead to progressive fibrosis, permanent alopecia and mottled areas of hyper- and/or hypo-pigmentation. Ulceration of the skin often occurs repeatedly after a single dose of radiation (Potten, 1985). The induction of cancer is the most critical outcome of exposure to radiation. In rats, tumor incidence correlates well with induction of cellular destruction and folicular atrophy (Burns, 1976). In humans, there is an increased number of skin carcinomas in patients who receive radiotherapy with cumulative incidences
ranging to 35 percent in patients with previous severe radiodermatitis. Overall, skin cancer rates are much higher in radiotherapy patients than in the unirradiated population. The absolute risk postulated for skin carcinomas induced by ionizing radiation is estimated to be between 0.4 and 1.0 cases per $10^6$ persons per cGy per year (Kohn and Fry, 1984). Seventy-five percent of all radiogenic skin tumors in humans are basal cell carcinomas. The remaining tumors are mostly squamous cell carcinomas. Basal cell carcinomas are usually not invasive, while squamous cell carcinomas exhibit a much higher risk of regional and metastatic involvement (Sanders and Kathren, 1983). In experimental animal models, basal cell carcinomas occur rarely except in rat skin exposed to ionizing radiation (Albert et al., 1969). In fact, experiments suggest that human and rat skin are approximately equal in their susceptibility to the carcinogenic action of x-rays when irradiated under comparable conditions. This is only true if allowances are made for the different times of expression of tumor development in the two species. The time ratio is similar to the ratio of life spans (Albert and Burns, 1976; Burns, 1986).

**Multi-Stage Mouse Skin Model**

Although the carcinogenic effects of ionizing radiation have been known for a long time, the mechanisms involved in cancer induction remain elusive. In an attempt to study the processes involved in radiogenic skin cancer, the mouse multi-stage model of
skin carcinogenesis was utilized in the experiments reported here. Most cancers develop as the result of a succession of steps occurring over a long period of time. An important feature of cancer progression in many human and animal tumors is the appearance of pre-malignant benign lesions, a small proportion of which undergo further changes and progress into malignancies (Boutwell, 1974; Scribner and Suss, 1978; Pierce and Fennell, 1982; Farber, 1984). Multifactorial, multistage models have been developed in a number of different species and tissues. In these model systems, carcinogenesis has been divided into initiation, promotion, progression and metastasis (Slaga, 1972; Armuth and Berenblum, 1974; Peraino, Fry and Grube, 1978; Day and Brown, 1980; Farber, 1980; Hicks, 1980; Patskan, et al., 1987).

The best characterized experimental animal model for tumor progression is based on mouse skin. In mouse skin, operational distinction between initiation and promotion has been clearly demonstrated. Initiation has been defined as a single subthreshold dose of a carcinogen and evidence indicates that it involves the induction of genetic alterations that are essentially irreversible. Promotion, on the other hand, involves repeated applications of a non-carcinogenic hyperplastic agent (Slaga, 1972; Boutwell, 1974; Diamond, O'Brien, and Baird, 1980) and may result in the clonal expansion of initiated cells. The initiation-promotion protocol in mouse skin produces predominantly papillomas and, to a much lesser degree, squamous cell carcinomas. Progression is the process by
which these benign papillomas develop malignant characteristics and is believed to involve a number of genetic alterations (Hennings et al., 1983; Hennings et al., 1986; O'Connell et al., 1986a, 1986b). Metastasis from squamous cell carcinomas of mouse skin rarely occurs and is thought to require further genetic change. Recently, Patskan et al. (1987) have reported a moderate-to-high incidence of lung metastasis in mice treated repeatedly with a subcarcinogenic dose of a carcinogen.

In initiation-promotion experiments using chemical initiators, although more than 90 percent of the squamous cell carcinomas develop from papillomas, the conversion rate has been shown to be rather low (Burns, 1978b). Recently, several investigators have treated mice bearing skin papillomas with tumor initiators and have observed an increase in carcinoma formation compared to control mice treated with the tumor promoter TPA (Hennings et al., 1983; O'Connell et al., 1986a, 1986b). These data suggest that malignant conversion requires additional steps, possibly a mutagenic alteration.

A number of laboratories have shown that the proliferative state of the target cell population can influence carcinogen-induced tumorigenesis. Increased rates of cell proliferation induced by hyperplastic agents prior to the application of a chemical initiator often result in a faster and greater response to the initiator (Pound, 1966, 1968; Hennings, Bowden and Boutwell, 1969; Hennings, Michael and Patterson, 1973; Bowden and Boutwell, 1974). In fact,
Hennings et al. (1969, 1973) have shown that the effect of TPA pretreatment on tumor incidence is dependent on the nature of the initiator. It has long been known that the sensitivity of cells to ionizing radiation varies with their age in the cell cycle and that the sensitivity of a tissue is directly proportional to the rate of cellular proliferation. An examination of the proliferative state of the skin prior to irradiation will aid in understanding mechanisms involved in initiation by ionizing radiation.

The effects of the duration of treatment with TPA on the number and incidence of skin papillomas and carcinomas as well as on the malignant conversion rate has been well documented for chemical initiators (Van Duuren et al., 1973; Verma and Boutwell, 1980; Hennings et al., 1983). Verma and Boutwell (1980) have shown that TPA promotion for 18, 24, 30 or 36 weeks elicited virtually identical yields of papillomas. The incidence of carcinomas on the other hand was less than maximal for mice promoted for 24 weeks or longer. In addition, Hennings et al. (1985) have shown that papillomas induced by the first few TPA treatments are much more likely to progress to carcinomas than those which appear later.

Taken together, these data imply that malignant conversion requires additional steps, possibly mutagenic alterations, besides those that induce benign papillomas. The mouse skin model was chosen for this study because: 1) induction of tumors has been shown to occur with both physical and chemical agents, and 2) it is possible to isolate tissue at several different defined biological
stages of tumorigenesis. This offers an opportunity to study progressive changes that occur at discrete stages of tumor development.

Ionizing Radiation - Experimental Evidence Related To Skin Tumor Induction in Rodents

Ionizing radiation has been shown to be a complete carcinogen in rodent skin following single and/or fractionated exposures. Several groups have shown that a carcinogenic dose of radiation increases the incidence of benign and malignant dermal tumors and squamous cell carcinomas. However, the incidence of papilloma formation in these studies was rare and the latency time for tumor formation was long (Hulse, 1967; Albert et al., 1969; Burns et al., 1978a). Most of the studies utilizing ionizing radiation have been designed on the premise that radiation is a complete carcinogen. Very few experiments have been designed to test the initiating and promoting effects of radiation separately. Animal studies have provided evidence which clearly indicates that ionizing radiation initiates events which are retained in viable cells for long periods of time. In addition, experiments suggest that these lesions do not undergo further change or expression until a subsequent event is induced. Early work carried out by Berenblum and Shubik (1949) established that ionizing radiation was an effective initiator. Later, Berenblum and Trainin (1960) found that doses of x-rays that were nonleukemogenic resulted in an appreciable incidence of
leukemia if followed by treatments with urethane. They interpreted these results to indicate that ionizing radiation at dose levels insufficient for complete carcinogenesis was an effective initiator. Tar from cigarettes has also been used to enhance skin tumorigenesis initiated by β-rays (McGregor, 1976). Hoshino and Tanooka (1975) showed that the latent carcinogenic events induced by β-irradiation in the skin were expressed after treatment with 4-nitroquinoline 1-oxide even if treatment was started as long as 400 days after irradiation, indicating that ionizing radiation initiates events that are retained in viable cells for long periods. Over 30 years ago, Shubik et al. (1953) used an initiation-promotion protocol to treat mice with a single subcarcinogenic dose of radiation followed by croton oil promotion. At the end of 8 months, 35 percent of the animals had developed papillomas. There was no appearance of any other type of benign or malignant tumors. More recently, Stenback (1975) investigated the initiating potential of ultraviolet light (UV) using the mouse two-stage model. The results of his studies showed that UV light is effective as an initiator in mouse skin. Distinct differences were noted between a chemical initiator, dimethylbenzanthracene (DMBA) and UV irradiation. In animals initiated with UV irradiation and promoted with croton oil, the number of tumors was fewer, both in terms of total tumor bearing animals and total tumors as well as number of tumors per animal as compared to those treated with DMBA. The other major difference was that the latency period was significantly longer in animals.
initiated with UV light and promoted with croton oil. Once again, the majority of tumors formed were papillomas.

Humans are exposed to a multiplicity of factors in their environment, which may be involved in the multifactorial multistage nature of cancer. The mutagenic effects of radiation along with its wide use in the medical field have prompted the animal experiments described in Chapters 2, 3 and 4. Data presented in Chapters 2, and 3 provides information on the effect of TPA pretreatment on the tumor incidence in mice initiated with ionizing radiation. The discussion of these results is continued in Chapter 3, where the effect of promotion duration on the incidence of papillomas, carcinomas and malignant conversion rate was examined. The data presented in Chapter 4 provides information on the ability of ionizing radiation to increase the malignant conversion rate of papillomas induced by an initiation-promotion protocol utilizing a chemical carcinogen as the initiator.

Oncogenes: History

The belief that genetic damage is responsible for cancer has evolved from a diverse body of information (Bishop, 1987). Human epidemiological data has provided evidence for hereditary predisposition to some cancers (Lynch, 1976; Schmike, 1979; Ponder, 1980). It has also shown a connection between inherited recessive diseases which impair the ability of cells to repair damaged DNA and susceptibility to cancer development (Lehman, 1982; Hanawalt and
Sarasin, 1986). Specific and nonspecific chromosomal abnormalities have been found in a wide range of malignant cells (Rowley, 1983; Yunis, 1983; Rowley, 1984). In addition, there is also a wide body of evidence relating mutagenic potential of a substance with its carcinogenic potential (Ames, 1979). Perhaps the most intriguing development has been the discovery of cellular proto-oncogenes.

The development of tumors in humans and in a number of experimental animal models occurs through complex multistep processes which can occupy over one half of the life span of the organism. Data predicts that multiple mechanisms are involved in the conversion of normal cells to fully malignant tumor cells. The development of new scientific techniques in the past eight years has made it possible to unveil the molecular events involved in neoplasia. Recent studies of the acute transforming retroviruses and DNA transfection procedures have identified at least 40 oncogenes of viral and cellular origin (Bishop, 1983; Vande Woude et al., 1984; Bishop, 1985) and have revolutionized experimental approaches used to study the genetic basis of multistage carcinogenesis.

The concept that there are genes capable of causing cancer (oncogenes) is largely based on studies carried out with transplantable tumors in chickens. In 1911, Rous described a transmittable sarcoma in chickens. The tumor in the adult bird could be transmitted horizontally to other chickens by injection of tumor cells or by injection of cell-free filtrates of the tumor.
Later, similar findings were observed in tumors arising in rats, mice and hamsters. The causative agent for such tumors was found to be a class of RNA viruses (type C retroviruses). Once oncogenes in RNA tumor viruses were recognized, a number of investigators sought to determine the origin of these genes. The major question was whether the genes had evolved wholly within the virus or had been acquired from another genome. The life cycle of the retroviruses provided the first clues. The single-stranded RNA of a diploid viral genome is transcribed into DNA and integrated into a host chromosomal DNA. The host cell then uses its own machinery to express the viral genes. Studies from a number of laboratories led to the discovery that sequences homologous to the oncogenic region of the retroviruses were present in DNA of all tissue examined in virus-free chickens and rodents. More extensive investigations have revealed that there are homologous sequences in yeast, drosophila, mice, and on up the evolutionary tree to humans. The oncogenic sequences in RNA tumor viruses are thought to be the result of recombinational events between retroviral and cellular genomes. This results in the insertion of cellular genes in the viral genome and their subsequent activation (Varmus, 1982; Bishop, 1983; Nuss, 1985). The normal cellular sequences are referred to as proto-oncogenes (Bishop, 1983; Varmus, 1984) and have been shown to be true genes in that they code for proteins. There is a high degree of homology between viral oncogenes and the cellular proto-oncogenes both at the nucleic acid and protein levels. Those proto-oncogenes
whose products have been characterized are known to play a major role in growth control and/or differentiation. Alterations in structure, function or level of expression of these proto-oncogenes can convert them to oncogenes. The products of these activated forms may then cause abnormal growth, differentiation, and intercellular coordination, thus contributing to the abnormal behavior of tumor cells.

It is important to point out differences between viral and cellular oncogenes. The concept of oncogenes as defined by retroviruses is in conflict with the multistage nature of human malignancies and animal models of carcinogenesis. Acute retroviruses contain small regions within their genome whose expression is sufficient to trigger carcinogenesis (Bishop, 1983). This has never been observed with cellular oncogenes. In fact, quite the opposite has been found. Evidence suggests that, unlike the situation of the acute retrovirus more than one oncogene or genetic alteration is needed to produce cancer cells. Land, Parada and Weinberg (1983b) have shown that introduction of two oncogenes are required to obtain a transformed phenotype in primary rat embryo fibroblasts (REF). On the basis of their REF assay, oncogene complementation groups have been established. Multiple cellular oncogenes have also been found in some human tumor cell lines and primary tumors (reviewed in: Land, et al., 1983a; Barbacid, 1985, 1986). There are three basic differences between viral and cellular oncogenes which could account for their separate characteristics.
First, viruses introduce genetic sequences coding for foreign proteins into target cells; chemical and physical carcinogens in contrast must somehow disturb the function of normal cellular genes. Second, the viral oncogenes are driven by very powerful regulatory elements, which have the potential to override cellular regulatory mechanism. Third, the virus delivers its oncogene to a large number of cellular targets, some of which may be undergoing critical stages of growth or differentiation.

**DNA Transfection**

The technology of transferring DNA from one mammalian cell to another is termed DNA transfection (Athwal and McBride, 1977; Wigler et al., 1978; Graham and Vander Eb, 1983). This technology set the stage for the successful transmission of the malignant phenotype from tumors to normal cells (Shih et al., 1979). The most common way used to detect dominant acting transforming genes in tumors is to precipitate tumor DNA in the presence of calcium phosphate and apply it to recipient cells. In most cases, the recipient cells are a mouse fibroblastic cell line, NIH3T3. The precipitate is taken up by the cells and some of the DNA is transferred to the nucleus where it is eventually integrated into the host genome. Following transfection, cells can be assayed for the development of transformed phenotype by a number of techniques. The focus assay selects for cells that have the ability to grow in the presence of low serum concentrations. Transformed cells are not contact
inhibited and will continue to grow. Foci of transformed cells appear on a background of contact-inhibited fibroblasts. Transformed cells can also be selected for their anchorage-independent growth. In this situation only transformed cells are capable of growing without adherence to a substrate when suspended in semisolid medium. Tumor formation is also used to select for transformed cells. Normal fibroblasts will not form tumors in syngeneic or immunologically deficient animals. Transformed fibroblasts will do so to varying degrees. The transfection assay can provide highly variable results due to a number of factors. The growth potential of the recipient cells is critical in the outcome of the assay. Also, the nature and quality of the DNA precipitate is extremely important. It is interesting to note that not all oncogenes are active in the mouse fibroblast established cell line. In addition, the use of gene transfer to detect oncogenes has been criticized because the recipient cells used in this assay are themselves abnormal. Whatever the assay's idiosyncrasies, gene transfer has provided a sensitive device to select for genes whose products appear to be involved in cancer development. The utility of the assay will grow as the recipient cells are diversified.

Gene transfer assays have revealed that approximately 20 percent of human tumor cell lines and tumor biopsies possess dominant transforming activity which can be detected in NIH3T3 cells (Varmus, 1984; Barbacid, 1985). Over 80 percent of the transforming genes detected belong to the ras family of oncogenes (Chang et al.,
1982; Der, Krontiris and and Cooper, 1982; Parada et al, 1982; Santos et al, 1982, 1984). The ras family of genes contains Ha-ras and Ki-ras, homologous to the Harvey and Kirsten murine sarcoma viral oncogenes (Ellis et al, 1981). It also contains N-ras, isolated from a neuroblastoma. A retroviral homolog of N-ras has not been found. The Ha, Ki and N-ras genes all encode proteins with a molecular weight of 21 Kd and are very homologous. These proteins are referred to as "p21's." They are each located in the cytoplasm adjacent to the plasma membrane and their precise functions are unknown. It is known, however, that they bind both guanosine tri- and di-phosphate, have guanosine triphosphatase activity, and are believed to be involved in transmembrane signal transduction. Gene transfer in NIH3T3 cells has also revealed a diverse array of oncogenes other than ras; B-lym, met and neu (Diamond et al, 1983; Cooper et al., 1984; Bargmann, Hung and Weinberg, 1986a). These genes have not been as extensively characterized as the ras genes. The recent identification of tumors which possess a dominant transforming activity which is not related to any of the known oncogenes is very intriguing (Fox and Watanabe, 1985; Garte et al., 1985; Reynolds et al., 1986;). As the recipient cells are diversified, one would predict an increase in identification of non-ras transforming genes.

Functional Role of Proto-Oncogenes

In multicellular organisms the behavior of individual cells must be highly coordinated with that of others. Increasing evidence
is emerging which indicates that proto-oncogenes code for proteins which are involved in development, growth and/or differentiation. More than 40 different cellular and viral oncogenes have been identified. They have been grouped into several categories based on the location of their encoded protein (plasma membrane, cytoplasmic or nuclear) and activities of their gene products. Proto-oncogene gene products have been found to function as external signals for cell proliferation (e.g., c-sis) as well as membrane receptors (e.g., c-erbB, c-fms, c-neu). They have also been shown to act as second messengers, transferring growth signals to the nucleus (e.g., the src family and ras family). The nuclear proto-oncogene products (c-fos, c-myb, c-myc, c-ski) are believed to have important roles in control of cell division (Weinberg, 1985).

There are a few biochemical mechanisms by which most of the proto-oncogene products appear to function. Perhaps the largest group fall into the category of protein kinases. This category of proto-oncogene products includes membrane-bound receptors and proteins located in the cytoplasm thought to be involved in signal transduction. They use either tyrosine as the substrate amino acids. Their activity is postulated to evoke a cascade of events which ultimately lead to alterations in growth control (Hunter, 1985; Hunter and Cooper, 1985). As discussed earlier, the ras family of gene products have the ability to bind guanosine triphosphate in a manner similar to G and N proteins (Hurley, 1984). There is a class of proto-oncogene products that affect gene
expression by influencing the biogenesis of mRNA (Kingston, Baldwin and Sharp, 1985; ), and a class that function as growth factors (Waterfield, 1985).

For a generalized discussion of the function of these proteins and how disruption of their normal function could result in a transformed phenotype, the proto-oncogene products can be broadly classified into two categories: those that reside in the cytoplasm and those that reside in the nucleus. Cytoplasmic proto-oncogene products (protein kinases and GTP binding proteins) are expressed in relatively constant amounts throughout the cell cycle. Their expression may vary somewhat depending on the growth and differentiation state of the cell. Weinberg (1985) has postulated that cytoplasmic proto-oncogene products are in a resting state awaiting a direct stimulus from an appropriate agonist. They then rise to an "excited" state, send an excitatory signal for a short period of time and fall back to relative inactivity. Negative feedback mechanisms would be required to limit the excited state (e.g., guanosine triphosphatase activity of ras or internalization and phosphorylation of receptor proteins).

The nuclear protein products function in a different manner from the cytoplasmic proteins. In response to growth stimulatory signals, there is an increase in the steady state concentration of the encoded nuclear proteins (Kelly et al., 1983; Cochran et al., 1984; Greenberg and Ziff, 1984; Muller et al, 1984). Alterations of steady state levels can be achieved by modifying the rate of
transcription, post-transcriptional and post-translational processing. The genetic lesions that create nuclear oncogenes have been shown repeatedly to result in constitutive expression of these genes by uncoupling them from their normal regulation.

There are at least three sites in growth control pathways at which oncogenic proteins might intercede to deliver a growth stimulus. The protein itself might mimic a growth factor, stimulating growth in an autocrine fashion (Hunter, 1985). Other proteins might imitate an occupied growth factor receptor and provide a mitogenic signal in the absence of exogenous growth factors. Third, the proteins might also act in intracellular growth control pathways (in either the cytoplasm or nucleus) uncoupling them from the need for an exogenous stimulus.

**Mechanism of Activation**

Proto-oncogenes are normal cellular genes that have the ability to contribute to the development of a malignant cell if their function or expression is altered through mutation, translocation, amplification or some other mechanism. Perhaps the most widely studied oncogenes are the ras genes. These genes are most commonly activated by alteration of the protein product through point mutations (Reddy et al., 1982; Santos et al., 1982; Tabin, Bradley and Bargmann, 1982; Taparowsky et al., 1982; Capon et al., 1983; Yuasa et al., 1983). Mutations in codons 12, 13, 59 or 61 resulting in single substitutions in the corresponding amino acid...
have been commonly found in the activated form of these genes (Reddy et al., 1982; Tabin et al., 1982). These structural alterations do not have any effect on the rate of expression. In fact, the only biological difference between the proto-oncogene and oncogene products found to date is a decrease in guanosine triphosphatase activity in the activated protein. This decrease in activity, however, does not correlate well with the transforming potential of the genes. So, until further knowledge of the functional role of these genes is attained, an understanding of how alterations in the structure of the protein confer transforming activity remain elusive.

A number of mechanisms other than point mutations within oncogene proteins have been implicated in the development of malignancy. Overexpression of the proto-oncogene can be a major mechanism of activation. This can be induced in several ways. Acquisition of a novel transcriptional promoter may result in an increase in expression (Blair et al., 1981). In vitro, this has been shown with the mos and Ha-ras proto-oncogenes: experimental addition of a strong transcriptional promoter results in the oncogenic activation of these two cellular genes (Blair et al., 1981; DeFeo et al., 1981; Spandidos and Wilkie, 1984). In vivo, both the c-myc and c-erbB avian proto-oncogenes have been shown to induce hematopoietic neoplasias after adjacent integration of an avian leukosis proviral DNA segment close to either one of these genes. This places the cellular genes under control of a strong
viral promoter (Hayward, Neel and Astrin, 1981; Payne, Bishop and Varmus, 1982; Fung, Lewis and Crittenden, 1983).

Overexpression may result from amplification of the proto-oncogene or translocation resulting in deregulation of expression. Amplification of the proto-oncogene c-myc was first reported in HL-60 cells derived from a patient with acute promyelocytic leukemia (Collins and Groundine, 1982; Alitalo et al., 1983). Amplified c-myc has also been found in cell lines derived from small-cell carcinoma of the lung. Here amplification appears to correlate with biological subtypes of the carcinoma showing aggressive clinical behavior. Amplification of c-myc and Ha, Ki and N-ras have all been reported for a variety of tumor types (Little et al., 1983; McCoy et al., 1983; Schwab et al., 1983a, 1983b; Alitalo, 1985). The role of amplified proto-oncogenes in the development of a tumor and its subsequent biological behavior is not known. It is assumed that the amplified genes convey a selective growth advantage to the cell. Events resulting from amplification may be important in the development of the tumor and/or in secondary changes associated with progression.

Chromosome translocations occur at a high frequency in some types of tumors suggesting a role in tumor development (Klein, 1981). Recently several genes situated near sites of common translocations have been identified. Many of these genes are known proto-oncogenes, supporting the hypothesis that chromosomal breaks might result in activation of proto-oncogenes located close to a
break point (Klein, 1981; Groffen et al., 1984; Rowley, 1984; Konopka et al., 1985). DNA rearrangement by chromosomal transposition can bring a proto-oncogene under cis-acting control of a highly active chromosome region. The most extensively studied systems are Burkitt's lymphoma and murine plasmacytomomas (Klein, 1983; Croce, 1986). The cellular myc gene has been shown to be translocated at high frequency in these two neoplasms. Normal cellular myc appears to be involved in control of differentiation and proliferation. Expression of the gene is tightly regulated through the cell cycle. In cells where there is a translocation, the myc gene has been found to be constitutive expressed, possibly contributing to the development of the malignant phenotype. Translocations also result in the production of new chimeric proteins with different activity than their normal counterpart. This occurs due to the production of mRNA from a fusion between the two translocated genes. An example of this is seen in chronic mylogenous leukemia with the activation of abl (Konopka and White, 1985; Konopka et al., 1985; ).

Support for a role of alteration of gene expression by amplification or translocation in the development of malignancy is supplied by data from in vitro studies. A number of investigators have shown that the spectrum of malignant properties induced by the ras oncogenes can be modulated by their level of expression (Land, Parada and Weinberg, 1983a; Ruley, 1983; Spandidos and Wilkie, 1984). Low levels of expression of activated ras transform rodent
fibroblast cell lines, while higher levels are required to transform primary rat embryo fibroblasts (REF). Also, normal ras fails to transform rodent fibroblasts, although, if the promoter is altered so that high levels of the normal ras protein are present, transformation does occur (Chang et al., 1982).

**Role of Oncogenes in Carcinogenesis**

The first human cellular oncogene to be identified was Ha-ras from a bladder carcinoma. Since then activated proto-oncogenes have been found in almost every histological form of human cancer (Pulciani et al., 1982; Eva and Aaronson 1985; Shimizu et al., 1985; Croce, 1986; Martin-Zanca, Hughes and Barbacid, 1986) and human tumor cell lines (Blair et al., 1982; Cooper et al., 1984; Fasano et al., 1984). The predominant activated proto-oncogenes found in human tissues belong to the ras gene family (Barbacid, 1985, 1986). The overall incidence of activated ras (Ha, Ki and N) is approximately 20 percent. However, activated ras genes do not correlate with any histopathological properties of the tumors making it difficult to assess their role in human tumor development. Other human oncogenes identified by different experimental approaches have been found to be altered in specific tumor types. The cellular raf-1 has been identified in gastric carcinomas (Shimizu et al., 1985) and HER-2/neu has been identified in breast cancers (Slamon et al., 1987). Both of these oncogenes show some degree of correlation between level of their expression and clinical staging of the
disease. The most intensely studied human cancers in terms of their cytogenetics and molecular biology are the leukemias and lymphomas (Croce, 1986). In B and T cell neoplasms, translocations of the proto-oncogene myc is a common event. This places the coding sequences of myc adjacent to elements capable of elevating gene transcription in a tissue-specific fashion (Klein, 1983). In chronic myelogenous leukemia, the translocation results in a chimeric gene (abl) leading to the expression of an altered gene product (Heisterkamp et al., 1983; Shtivelman et al., 1985). Although activated myc and abl have been established in human hematopoietic tumors the question still remains: Which came first, the translocation resulting in activation of the cellular oncogenes or the neoplasia?

In an attempt to address the question of the role of oncogenes in tumor development, a number of animal model systems have been studied. As seen in Table 2, activated proto-oncogenes have been identified in a wide variety of tumor types induced by many different chemical and physical carcinogens in experimental animals. The predominant oncogenes found are Ha and Ki-ras. Experimental evidence in rats and mice suggest that ras oncogenes are involved in the tumor initiation process (Balmain et al., 1984; Zarbl et al., 1985; Quintanilla et al., 1986). There is an 86 percent incidence of activated Ha-ras in rat mammary tumors induced by a single dose of methylnitrosourea (NMU) during sexual development (Zarbl et al., 1985). NMU is a direct-acting alkylating agent with
a short half life. The activated Ha-ras genes in all tumors studied contained the same point mutation, a GC to AT transition at the 12th codon. This particular mutation happens to be predicted on the basis of direct interaction between NMU and cellular DNA. The interaction results in alkylation of deoxyguanosine residues (resulting in the production of 0-6-methyl deoxyguanosine), leading to mispairing during replication and the eventual formation of a transition mutation (Sukumar et al., 1983; Zarbl et al., 1985). This finding is highly suggestive of a role of activated Ha-ras in the induction of mammary carcinomas in rats. Balmain and colleagues (1984; Quintanilla et al., 1986) have found activation of Ha-ras in 89 percent of mouse skin tumors induced by initiation with DMBA plus TPA promotion. The activation of Ha-ras did not correlate with malignant tumor development since premalignant papillomas had the same transforming ability as invasive carcinomas. This is in agreement with the current hypothesis that additional genetic alterations are required to transform papillomas into carcinomas (Hennings et al., 1983; O'Connel et al., 1986a,1986b). Further support for this hypothesis comes from Balmain's group. They have recently found that while papillomas are usually heterozygous for the ras mutation, some squamous cell carcinomas are homozygous and the mutated allele is amplified (Quintanilla, et al., 1986).

Interestingly, it has recently been shown that direct application of Harvey murine sarcoma virus to mouse skin can replace the need for chemical initiators (Brown, et al., 1986). Ras retroviruses have
Table 2.
Proto-oncogene Activation in Animal Tumor Models

<table>
<thead>
<tr>
<th>Species</th>
<th>Oncogene</th>
<th>Type of Cancer</th>
<th>Carcinogen</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>Ha-ras</td>
<td>Skin</td>
<td>dimethylbenzanthracene (DMBA) plus TPA</td>
<td>Balmain et al. 1984, Quintanilla et al., 1986</td>
</tr>
<tr>
<td></td>
<td>(33/37)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ha-ras</td>
<td>Skin</td>
<td>DMBA</td>
<td></td>
<td>Bizub, Wood &amp; Skalka, 1986</td>
</tr>
<tr>
<td>(3/4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ha-ras</td>
<td>Skin</td>
<td>dibenzacridine (DB ACR)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(5/6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ha-ras</td>
<td>Mammary</td>
<td>DMBA</td>
<td></td>
<td>Dandekar et al., 1986</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ki-ras</td>
<td>Fibrosarcoma</td>
<td>3-methylcholan-</td>
<td>Eva and Aaronson, 1983</td>
<td></td>
</tr>
<tr>
<td>(2/4)</td>
<td></td>
<td>threne (3MC)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ki-ras</td>
<td>Thymic</td>
<td>3MC</td>
<td></td>
<td>Eva and Trimmer, 1986</td>
</tr>
<tr>
<td>(10/12)</td>
<td>lymphomas</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ki-ras</td>
<td>Thymic</td>
<td>X-rays</td>
<td></td>
<td>Guerrero et al., 1984a</td>
</tr>
<tr>
<td>(4/7)</td>
<td>lymphomas</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-ras</td>
<td>Thymic</td>
<td>methyl nitroso-</td>
<td>Guerrero et al., 1984b, 1984c</td>
<td></td>
</tr>
<tr>
<td>(5/6)</td>
<td>lymphomas</td>
<td>urea (NMU)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ha-ras</td>
<td>Hepatomas</td>
<td>hydroxyacetyl-</td>
<td>Weiseman et al., 1986</td>
<td></td>
</tr>
<tr>
<td>(7/7)</td>
<td></td>
<td>aminofluorene</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ha-ras</td>
<td>Hepatomas</td>
<td>vinyl carbamate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(7/7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ha-ras</td>
<td>Hepatomas</td>
<td>hydroxydehydro-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(11/11)</td>
<td></td>
<td>estragole</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ha-ras</td>
<td>Hepatomas</td>
<td>diethylnitrosamine (DEN)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(7/14)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Species</td>
<td>Oncogene</td>
<td>Type of Cancer</td>
<td>Carcinogen</td>
<td>Reference</td>
</tr>
<tr>
<td>---------</td>
<td>----------</td>
<td>----------------</td>
<td>------------</td>
<td>--------------------</td>
</tr>
<tr>
<td>Rat</td>
<td>Ki-ras</td>
<td>Skin</td>
<td>ionizing radiation</td>
<td>Sawey et al., 1987</td>
</tr>
<tr>
<td></td>
<td>myc</td>
<td>mammary</td>
<td>NMU</td>
<td>Zarbl et al., 1985</td>
</tr>
<tr>
<td>Ha-ras</td>
<td></td>
<td></td>
<td>DMBA</td>
<td>&quot;</td>
</tr>
<tr>
<td>(61/71)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ha-ras</td>
<td></td>
<td>Fibrosarcoma</td>
<td>dinitropyrene</td>
<td>Tahira et al., 1986</td>
</tr>
<tr>
<td>(6/29)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ki-ras</td>
<td></td>
<td>Mesenchymal</td>
<td>methyl-methoxy methyl nitrosamine</td>
<td>Sukumar et al., 1986</td>
</tr>
<tr>
<td></td>
<td></td>
<td>kidney</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ki and</td>
<td>Neu</td>
<td>Neuroblastoma</td>
<td>ethylnitrosourea (ENU)</td>
<td>Schechter et al, 1984</td>
</tr>
<tr>
<td>N-ras</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>?</td>
<td></td>
<td>nasal</td>
<td>methyl methane-sulfonate</td>
<td>Garte et al., 1985</td>
</tr>
<tr>
<td>Ki-ras</td>
<td></td>
<td>liver</td>
<td>Aflatoxin</td>
<td>McMahon et al., 1984</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
also been shown to block new born mouse keratinocytes in phorbol ester-responsive, early stage terminal differentiation (Yuspa, et al., 1985). In vitro studies, as stated earlier show that ras is necessary but not sufficient to elicit malignant transformation (Land, et al., 1983b; Dotto, Parada, and Wienberg, 1985; Wienberg, 1985). The in vivo and in vitro data taken together suggest that ras oncogenes can play a role in initiation of carcinogenesis if cells harboring ras oncogenes are induced to proliferate (i.e., external application of tumor promoters or normal physiological processes such as mammary gland differentiation).

Other evidence which suggests that a single cellular oncogene can contribute to, but is not sufficient to confer, full tumorigenic properties on normal cells comes from studies of the myc gene family. There are three known myc loci: c-myc, N-myc and L-myc. They are all located on different chromosomes. There is significant sequence homology both at the nucleic acid and amino acid level between the three genes and their products. All encode nuclear proteins and have been found to be overexpressed in a variety of human and animal cancers (Alitalo, 1983; Schwab et al., 1983b; Brodeur et al., 1984; Lee, Murphree and Benedict, 1984; Nau et al, 1985). Recently transgenic mice have been generated carrying c-myc driven by the mouse mammary tumor virus-LTR (MMTV) or by the \( \mu \) and \( \chi \) promoters of the immunoglobulin genes (Adams et al., 1985; Leder et al., 1986). Some of the transgenic mice carrying c-myc driven by MMTV-LTR developed mammary carcinomas. The level of expression of
the chimeric c-myc gene was the same in all breast tissue regardless of tumor formation. In the strain of transgenic mice containing the chimeric c-myc driven by $\mu$ or $\chi$ promoters, most animals developed B-cell leukemias. However, most leukemias were clonal, indicating that the presence of constitutively active c-myc is not enough for leukemogenesis. This illustrates that activation of cellular oncogenes contributes to the carcinogenic process, but tumor progression involves rather extensive genomic alterations.

In addition to proto-oncogenes, there exists a reciprocal set of genes whose products inhibit cell growth and/or induce cells to undergo terminal differentiation. These two sets of genes are needed to achieve the complex control that is required for normal growth, development and differentiation. Mutations that result in activation of proto-oncogenes have a dominant effect on growth. Mutations that inactivate growth inhibitor or differentiation genes also result in growth stimulation. These mutations, however, should be recessive since it is necessary to inactivate both functional alleles for tumors to occur (Commings, 1973; Koufos, et al., 1984; Stanbridge, 1984; Green and Wyke, 1985; Knudson, 1985; Sachs, 1986; Sager, 1986). Although tumors could result from either mechanism, it seems most likely that tumors result from a distortion in the net balance in function of both types of gene products.

In spite of the enormous progress during the last five years in the field of molecular carcinogenesis, much remains to be learned. Oncogenes have not been identified in many types of human
cancers and specific oncogenes are not uniformly amplified or expressed in any given type of human cancer (Cooper, 1982; Slamon et al., 1987). The study of tumor suppressor genes (Stanbridge, 1984; Sager, 1986) is still in its infancy. In spite of all the knowledge that has been obtained, one must keep in mind that carcinogenesis results from a combination of genetic and epigenetic alterations affecting the capacity of cells to overcome their normal proliferate and differentiation restraints and to escape immuno-surveillance.

The research presented in this dissertation addresses some fundamental aspects of radiation carcinogenesis. Despite extensive studies examining the complete carcinogenic effects of ionizing radiation, very few in vivo experiments have been designed to test the initiating and promoting effects of ionizing radiation separately. Data presented in Chapter 2 provides information on the initiating potential of ionizing radiation in mouse skin. The discussion of these results is continued in Chapter 3 where the proliferative state of the skin prior to irradiation and promotion duration after irradiation was examined. The experiment described in Chapter 4 was designed to test the hypothesis that ionizing radiation may be highly active in malignant progression. Data generated from the experiments presented in Chapters 2, 3 and 4 will aid in understanding possible mechanisms involved in radiogenic-induced cancers at a cellular/tissue level. Data presented in Chapter 5 provides information on detection and characterization of dominant transforming genes in these induced tumors.
CHAPTER 2

IONIZING RADIATION AS AN INITIATOR IN THE
MOUSE TWO-STAGE MODEL OF SKIN TUMOR FORMATION

Introduction

Ionizing radiation has been shown to be a complete carcinogen in rodent skin after either single or fractionated exposures. Several groups have shown that X-rays increase the incidence of benign and malignant dermal tumors and squamous cell carcinomas. However, benign epithelial tumors were rare and the latency time for tumor formation was very long (Hulse, 1967; Albert et al., 1969; Burns et al., 1978a).

The cocarcinogenic effects of ionizing radiation on rodent skin and in a variety of other animal models have been extensively investigated (Cloudman et al., 1955; Shellabarger, 1967; Hoshino and Tanooka, 1975; Fry et al., 1976; McGregor, 1976; Arseneau, Fowler and Bakemeier, 1977; Lurie, 1977; Ullrich, 1980). In 1976, McGregor demonstrated that tar from cigarettes could enhance skin tumorigenesis in rats which had been initiated with $\beta$-irradiation (McGregor, 1976). Hoshino and Tanooka (1975) used $\beta$ particles and 4-nitroquinoline-N-oxide (4-NQO) to show that ionizing radiation induced alterations that are stable in viable cells for long periods without any evidence that the lesions undergo further change or
expression until a subsequent event is induced. These reports suggest that the carcinogenic effect of ionizing radiation can persist for long periods after the initial exposure. The essential event in radiation tumorigenesis may be subsequent to the initial radiation-induced lesion.

Despite many extensive studies, very few in vivo experiments have been designed to test the initiating and promoting effects of ionizing radiation separately. In this chapter, the mouse skin two-stage model of carcinogenesis was used to study the initiating potential of ionizing radiation. Over 30 years ago Shubik and colleagues (1953) used an initiation-promotion protocol to treat female Swiss mice with a single subcarcinogenic dose of radiation followed by croton oil promotion. At the end of 8 months, 35% of the animals had developed papillomas. No other type of benign or malignant tumor was reported. It was of interest to investigate whether subcarcinogenic doses of ionizing radiation induce tumors in mouse skin when followed by a promoting agent. The tumor promoter 12-O-tetradecanoyl phorbol-13-acetate (TPA) was used and its effect on tumor incidence was studied either when applied as a single dose prior to a subcarcinogenic dose of ionizing radiation or, as in the classical promotion protocol, multiple applications for an extended period after the initiating event. Histopathologic evaluations of tumor sections were performed to determine if tumors induced in this manner progress to malignancy. If ionizing radiation is an initiating agent in mouse skin, a promoting agent such as TPA should
enhance the incidence of tumors. The evidence presented here indicates that ionizing radiation can act as an initiator in the mouse two-stage model and that TPA promotion enhances this effect.

Materials and Methods

Female mice 5 to 7 weeks of age were used for these studies. Sencar mice were purchased from Harlan Sprague Dawley, Walkersville, Maryland. Five animals were housed in each stainless-steel cage in light and temperature controlled rooms. Food and water were available ad libitum. All animals were allowed at least 2 weeks to adapt to their new surroundings after arrival before experiments were undertaken. The dorsal skin of the mice was shaved 3 to 4 days before treatment, and only those mice in the resting phase of the hair cycle were used for experimentation.

TPA (purchased from Peter Borchert, Eden Prairie, MN) solutions were prepared in acetone and were administered to the shaved backs of individual mice in a volume of 0.2 ml. Control mice were treated with the same volume of acetone. Animals that were pretreated with TPA were painted with 17 nmol 24 hours prior to irradiation. TPA promotion was started 2 weeks after irradiation. Animals were treated twice per week with 8 nmol for a total of 60 weeks.

The mice were X-irradiated using a 4 MeV linear accelerator at a dose rate of 3.1 Gy per minute. A single fraction dose was delivered to an unopposed 2 x 8-cm field of dorsal skin. A source-
to-skin distance of 60 cm was used to achieve the desired dose rate. To irradiate only the dorsal skin, the animals were injected with Nembutal (50 mg/kg) and the skin was held away from the body (Fig. 1). To eliminate dosimetric uncertainty arising from skin sparing, the irradiated skin was bolused to a depth of 1 cm. Animals were irradiated with a total dose of either 7.5, 11.25 or 22.5 Gy. One half of all the animals receiving 7.5 Gy were pretreated with TPA prior to irradiation. The remaining half were pretreated with acetone as a control. Animals receiving either 7.5 or 11.25 Gy were promoted with TPA. Animals who received a dose of 22.5 Gy were not promoted.

All groups contained between 25 and 35 animals. During the entire period of study, cages were checked twice per week for dead or moribund animals. Once per week tumors were counted and measured and the location was noted. Due to the low incidence of tumors per animal, it was possible to follow individual tumors over the course of the study and to actually visualize their progression. Necropsies were performed at the time of termination. Tumor sections were fixed in 10% phosphate-buffered Formalin. Histopathological evaluations were made by Dr. J.N. Shively, Department of Veterinary Sciences, University of Arizona.

To investigate whether tumor incidence rates differed between treatment groups, the $x^2$ test was used (Snedcor and Cochran, 1980). Time to first tumor was compared between treatment groups using the log rank test (Peto et al., 1976; Peto et al., 1977) as per the
Figure 1. Schematic of the Irradiation Protocol.

The mice were irradiated with 4 MeV photons from a linear accelerator. A single fraction dose was delivered to an unopposed 2 x 8 cm field of dorsal skin. A source-to-skin distance of 60 cm was used for the desired dose rate (3.2 Gy/min). Animals were injected with Nembutal (50 mg/kg) and the dorsal skin held away from the body with forceps placed at the base of the neck and tail, so that the collimator jaws shielded the animal's body. To eliminate dosimetric uncertainty arising from skin sparing, the irradiated skin was bolused to a depth of 1 cm.
method Gail, Santer and Brown (1980). Statistical analyses were performed by Dr. Dalice Sim, Biostatistician, Director, Clinical Trials Resources Group, John P. Robarts Research Institute, London, Ontario.

Results

The results of the experiments involving use of ionizing radiation as an initiator followed by TPA promotion are depicted in Table 3. Seventeen percent of the animals in the control group receiving only TPA promotion developed tumors. Those animals that were irradiated with 11.25 Gy and then promoted with TPA had a total tumor incidence of 22%. The overall tumor incidence between these two groups of animals was not significantly different as measured by the $X^2$ test ($P > 0.05$). However, distinct differences in tumor type were observed between these two groups. The number of papillomas per mouse was higher in the control group receiving TPA alone, 0.23 vs 0.13. Of greater importance was the observation that only animals irradiated and promoted developed squamous cell carcinomas (6% of the animals). All squamous cell carcinomas were observed to arise from pre-existing papillomas with a malignant conversion rate of 33% and a median time of 27 weeks (data not shown).

Animals receiving 11.25 Gy followed by acetone treatment did not develop any papillomas or squamous cell carcinomas for 60 weeks. The incidence of other tumor types was virtually identical in the control group and the two groups irradiated with 11.25 Gy (3,3 and
Table 3.

Tumor Incidence in Sencar Mice Initiated with Ionizing Radiation and Promoted with TPA

<table>
<thead>
<tr>
<th>Treatment^a</th>
<th>% Animals with Tumors</th>
<th>Tumors per Mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total^b Papilloma</td>
<td>Squamous</td>
</tr>
<tr>
<td>X-ray Dose (Gy)</td>
<td>Promotion</td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>TPA</td>
<td>17</td>
</tr>
<tr>
<td>11.25</td>
<td>TPA</td>
<td>22</td>
</tr>
<tr>
<td>11.25</td>
<td>Acetone</td>
<td>8</td>
</tr>
<tr>
<td>22.5</td>
<td>---</td>
<td>9</td>
</tr>
</tbody>
</table>

^a Treatment sequences were as specified under Materials and Methods.

^b Numbers in parentheses represent the tumor-bearing animals divided by the total animal number.
4%, respectively). The other tumor types include fibrosarcomas, adenocarcinomas, and basal cell carcinomas (in order of highest to lowest incidence). The number of these tumors was too low for statistical evaluation.

The group of animals that received 22.5 Gy was not promoted. This dose resulted in definite radiation burns on Sencar mice. Hair loss, revealing red skin which frequently desquamated to yield a moist surface, was observed. Healing occurred relatively rapidly and was well underway during the third week. All mice receiving this dose retained a permanently epilated area which encompassed the entire irradiated field. TPA administration to the healed dorsum induced a recurrence of moist desquamation and if promotion continued led to severe ulceration. This occurred if promotion was started at any time between 2 to 20 weeks after irradiation. Animals in this group had a higher incidence of other tumors (9% vs 3-4%), as shown in Table 3. No epidermal tumors developed during the 60-week observation period. This finding is consistent with published results which show that dermal tumors occur prior to those of epidermal origin in mice receiving a single dose of ionizing radiation (Hulse, 1967).

Earlier studies report that the proliferative state of a target cell population can influence carcinogen-induced tumorigenesis. Several groups have shown that increased rates of cell proliferation induced by hyperplastic agents prior to the application of a chemical initiator often result in a faster and/or
greater response to the initiator (Pound, 1966; Pound, 1968; Hennings et al., 1969; Hennings et al., 1973; Bowden and Boutwell, 1974). In Table 4 the effects of TPA pretreatment on the initiating potential of ionizing radiation are illustrated. Animals that were pretreated, irradiated, and promoted had a total tumor incidence of 34%. This was statistically significant from all other treatment groups as measured by the χ² test (P < 0.001). Animals in the control groups, those receiving TPA promotion alone and those that were pretreated and irradiated only, had an overall tumor incidence of 17%. In the control group receiving TPA promotion, 13% of the animals had papillomas and 4% had other tumor types, while 17% of the animals receiving TPA pretreatment followed by irradiation had non-epidermal tumors only. The other tumor types were fibrosarcomas, adenocarcinomas, and basal cell carcinomas (in order of highest to lowest incidence). Animals receiving a single dose of TPA alone did not develop any tumors during the 60 weeks. Once again, it was observed that only animals that were irradiated and promoted developed squamous cell carcinomas. All squamous cell carcinomas arose from pre-existing papillomas except in one case. The malignant conversion rate for the group of animals pretreated, irradiated with 7.5 Gy, and promoted with TPA was 45% with a median time of 27 weeks (data not shown).

Comparison of Tables 3 and 4 shows that TPA pretreatment led to an increase in the incidence of squamous cell carcinomas as well as other tumor types. It was also noted that groups receiving
Table 4.

Effect of TPA Pretreatment on Tumor Incidence in Sencar Mice Initiated with Ionizing Radiation and Promoted with TPA

<table>
<thead>
<tr>
<th>Treatment</th>
<th>X-ray Dose(Gy)</th>
<th>Promotion</th>
<th>% Animals with Tumors</th>
<th>Tumors per Mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Total</td>
<td>Papilloma</td>
</tr>
<tr>
<td>TPA</td>
<td>--</td>
<td>--</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(0/20)</td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>TPA</td>
<td>17</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(6/30)</td>
<td></td>
</tr>
<tr>
<td>TPA</td>
<td>7.5</td>
<td>TPA</td>
<td>34</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(12/25)</td>
<td></td>
</tr>
<tr>
<td>TPA</td>
<td>7.5</td>
<td>Acetone</td>
<td>17</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(4/24)</td>
<td></td>
</tr>
</tbody>
</table>

a Treatment sequences were specified under Materials and methods.

b Numbers in parentheses represent tumor-bearing animals divided by total animal number.
a lower dose of irradiation plus TPA promotion developed more tumors than those receiving a higher dose and no pretreatment. In the incidence of other tumor types from Tables 3 and 4 was further catalogued into fibrosarcomas, adenocarcinomas, and basal cell carcinomas as shown in Table 5. As stated earlier, the number of these tumors was too low for statistical evaluation. The incidence of fibrosarcomas and adenocarcinomas was similar. The incidence of basal cell carcinomas, on the other hand, was much lower. There were only two basal cell carcinomas. One occurred in the group of animals pretreated with TPA, irradiated with 7.5 Gy, and promoted with TPA. This tumor was not associated with hair follicles. The other basal cell carcinoma arose in the group of animals that were treated with 22.5 Gy. This tumor was closely associated with hair follicles. The basophilic cells which made up the tumor closely resemble the hair matrix cells in the hair follicles.

Analysis of time to first tumor taking into account only the number of animals at risk for the two groups of animals irradiated with 11.25 Gy is shown in Figure 2. Log rank analysis of these two curves (P=0.15) indicates that TPA can enhance the effect of ionizing radiation on tumor production, but under these conditions the enhancement is not statistically significant.

Figure 3 shows a statistically significant different in the mean time to first tumor between the groups of animals pretreated, irradiated, and promoted with either TPA or acetone as analyzed by the log range test (p=0.004). Thus, under experimental conditions
<table>
<thead>
<tr>
<th>Treatment</th>
<th>X-ray Dose (Gy)</th>
<th>Promotion</th>
<th>Total Animal Number</th>
<th>Fibrosarcomas</th>
<th>Adenocarcinomas</th>
<th>Basal Cell Carcinomas</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPA</td>
<td>--</td>
<td>--</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>---</td>
<td>--</td>
<td>TPA</td>
<td>30</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>---</td>
<td>11.25</td>
<td>TPA</td>
<td>32</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>---</td>
<td>11.25</td>
<td>Acetone</td>
<td>25</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>---</td>
<td>22.5</td>
<td>--</td>
<td>35</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>TPA</td>
<td>7.5</td>
<td>TPA</td>
<td>35</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>TPA</td>
<td>7.5</td>
<td>Acetone</td>
<td>24</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>
Figure 2. Time to First Tumor Taking Into Account Only the Number of Animals at Risk.

Animals were irradiated with 4 MeV photons from a linear accelerator. A single dose of 11.25 Gy was delivered to a 2 x 8 cm field of dorsal skin. Two weeks after irradiation promotion was started. One half of the animals ([]) were promoted with TPA, 8 nmol in 0.2 ml acetone two times/week for 60 weeks. The other group of animals (0) were treated with acetone alone.
Figure 3. The Effect of TPA Pretreatment on Time to First Tumor Taking Into Account Only the Number of Animals at Risk.

All animals were painted with 17 nmol of TPA in 0.2 ml acetone 24 hours prior to receiving a single dose of 7.5 Gy to a 2 x 8 cm area of dorsal skin. Two weeks later one half of the animals (○) were promoted with TPA, 8 nmol in 0.2 ml acetone two times/week for 60 weeks. The other half (△) were treated with acetone alone.
where animals have been pretreated with a single dose of TPA 24 hours prior to irradiation, TPA promotion significantly enhances tumor incidence.

Comparison of Figures 2 and 3 shows that inclusion of TPA pretreatment did not alter the mean time to first tumor in those animals that were irradiated and promoted. As in the group that was initiated and promoted only (11.25 Gy plus TPA, Figure 2), tumor-bearing animals harbored one to three tumors. However, more animals developed tumors after TPA pretreatment (TPA plus 7.5 Gy plus, TPA, Figure 3).

In summary, only animals that were promoted developed papillomas. The papillomas were ordered in structure with many features of normal epidermis remaining in each group. Papillomas occurred after a long latent period, approximately 33 and 23 weeks for the TPA control group and the two groups that were irradiated followed by promotion, respectively. Only those groups of animals that were irradiated followed by TPA promotion developed squamous cell carcinomas. It appears that both TPA pretreatment and promotion led to a decrease in the time to first tumor and that TPA pretreatment led to an overall increase in tumor incidence.

Discussion

These experiments indicate that ionizing radiation is effective as an initiator in mouse skin. The results confirm earlier findings that ionizing radiation is a weak initiator
(Shubik, et al., 1953; Hulse, 1967; Stenback, 1975). The number of tumors which develop in animals receiving X-irradiation plus TPA is smaller than that reported in the literature for chemical initiators plus TPA. This is true both in terms of total tumor-bearing animals and in the number of tumors per animal. Additionally, in contrast to tumors induced experimentally by potent chemical or oncogenic viruses, ionizing radiation-induced tumors have been shown previously to have long latent periods (Shubik, et al., 1953; Boutwell, 1974; Stenbnack, 1975; Slaga and Fisher, 1983; Reiners, Nesnow and Slaga, 1984). The results presented in this chapter agree with this finding (Figures 2 and 3).

The efficacy of TPA as an enhancer of radiation-induced tumorigenesis in these experiments is consistent with the results of Shubik et al. (1953) and demonstrates that papillomas will progress to malignancy after increased exposure to the promoter. More recently, Fry and colleagues (R. J. M Fry, S. Ogle, and L. Triplett,). Experimental induction of skin cancer with x-rays, Abstract Hg-4, Radiation Research Socity, 33rd Annual Meeting, 1985) have shown that promotion of skin (female SKH:hairless-1 mice) which has been initiated with X-rays results in a substantial increase in the incidence of squamous cell carcinomas. In this study it was also noted that the animals had a higher incidence of carcinomas which were not observed to arise from pre-existing benign lesions. This discrepancy is most likely a function of strain differences. Sencar mice were bred selectively for their sensitivity to skin
tumor induction by dimethyl-benzanthrene (DMBA) initiation followed by TPA promotion. Consequently, the Sencar mouse is extremely sensitive to two-stage carcinogenesis and to TPA promotion and thus has a propensity for papilloma formation (Reiners et al., 1984).

Epidermal papillomas are the most frequently observed type of neoplastic lesion produced in mouse skin as a result of initiation and promotion. The frequency of conversion of papillomas to malignant lesions has been the subject of controversy. There is evidence in the literature that at least some papillomas are precursors of carcinomas (Friedewold and Rous, 1950; Shubik et al., 1953; Train, 1967; Boutwell, 1974). Burns et al. (1978b) have indicated that although more than 90% of epidermal carcinomas develop from papillomas, the conversion rate of the papillomas is very low (5 to 7%). A malignant conversion rate of 33% for animals initiated and promoted and a 45% conversion rate for those which were pretreated with TPA prior to initiation and promotion was observed in these studies. Burns's group used DMBA initiation followed by TPA promotion and found that 80% of the induced papillomas were conditional and required the continued presence of the promoter. The fact that conditional papillomas probably contribute very little to the carcinoma yield, and that only the first carcinoma on a given animal was used in their study when calculating conversion rate, might account for the large rate difference in malignant conversion of papillomas between this work and that of Burns. One could also speculate that
fundamental differences in the mechanism of action of X-rays and DMBA may account in part for the observed differences in malignant conversion rates. It would be of interest to determine the effect of promotion duration on the incidence of papillomas and carcinomas using ionizing radiation as an initiator.

TPA pretreatment led to an increase in tumor incidence with no apparent effect on tumor induction time. Although it has been demonstrated that pretreatment with a hyperplastic agent results in a higher incidence of epidermal tumors (Pound, 1966; Pound, 1968; Hennings et al., 1969; Hennings et al., 1973; Bowden and Boutwell, 1974), it was a surprise to observe that TPA pretreatment led to an increase in tumors of non-epidermal origin as well as carcinomas (Table 4). It is well documented that an acute dose of TPA exerts many effects on the epidermis: (1) stimulation of DNA synthesis, (2) stimulation of RNA and protein synthesis, (3) induction of inflammation and influx of leukocytes, and (4) stimulation of cell division (reviewed in: Argyris, 1980). The assumption that an alteration in DNA is necessary for initiation suggests potential mechanisms by which an increased rate of DNA synthesis prior to irradiation could lead to an increased frequency of initiation, but at present the reasons for this are not known. It is somewhat more difficult to explain how TPA pretreatment could result in an increased incidence of non-epidermal tumors. A recent study by Aldaz et al. (1985) has shown that the interfollicular epidermis is not the only target of TPA. The hair follicles as well as the
dermal components are affected by TPA treatment. Modification of
the whole skin by TPA could, in some as yet uncharacterized fashion,
account for the increase in the total tumor incidence in animals
that were pretreated prior to irradiation.

In summary, at the doses examined here, radiation is shown to
function as an initiator in mouse skin. These subcarcinogenic doses
of ionizing radiation require TPA promotion for papilloma formation
and subsequent malignant conversion. Tumor incidence can be
modified by TPA pretreatment.
CHAPTER 3

IONIZING RADIATION AS AN INITIATOR: EFFECTS OF PROLIFERATION AND PROMOTION TIME ON TUMOR INCIDENCE

Introduction

Animal studies have provided evidence that clearly indicates that ionizing radiation initiates events which are retained in viable cells for long periods of time. There is no evidence that the induced lesion(s) undergo further change or expression until a subsequent event is induced. Hoshino and Tanooka (1975) showed that the latent carcinogenic events induced by $\beta$-irradiation in the skin were expressed after treatment with 4-nitroquinoline 1-oxide. This occurred even if treatment was started as long as 400 days after irradiation. Early work carried out by Berenblum and Shubik (1949) established that ionizing radiation was an effective initiator. Shubik and colleagues (1953) found that exposure of mouse skin to $\beta$-rays, which alone produced no tumors during the course of the experiment, did so if the animals were promoted with croton oil. In Chapter 2 of this dissertation data is presented which shows that X-rays can act as an initiator in Sencar mouse skin and that TPA promotion enhances this effect. These studies have been expanded in CD-1 mice to further investigate the effect on tumor incidence of the proliferative state of the skin prior to irradiation and
promotion duration after irradiation.

A number of laboratories have shown that the proliferative state of the target cell population can influence carcinogen-induced tumorigenesis. TPA pretreatment causes a loss of basal cells and an increase in the number of suprabasal cells (Argylis, 1980; Reiners and Slaga, 1983). The loss of basal cells is followed by a peak of DNA synthesis at approximately 30 hours and a peak of mitosis at 2 days (Argylis, 1980). The increased rates of cell division are achieved by a reduction in the cell cycle time from 5 to 7 days prior to TPA pretreatment to 16 hours at the peak of TPA-induced proliferation. The increased rates of cell division are accompanied by a reduction in epidermal transit time (Morris and Argylis, 1983). Increased rates of cell proliferation induced by hyperplastic agents prior to the application of a chemical initiator often result in a faster and greater response to the initiator (Pound, 1966; Pound, 1968; Hennings et al., 1969; Hennings et al., 1973; Bowden and Boutwell, 1974). In fact, Hennings et al. (1969; 1973) have shown that the effect of TPA pretreatment on tumor incidence is dependent on the nature of the initiator. Thus, it was of interest to examine the effect of TPA pretreatment on the tumor incidence with ionizing radiation as an initiator.

The effects of the duration of the treatment with TPA on the number and incidence of skin papillomas and carcinomas as well as the malignant conversion rate has been well documented for chemical initiators (Burns et al., 1978b; Van Duuren et al., 1973; Verma and
Verma and Boutwell (1980) have shown that TPA promotion for 18, 24, 30 or 36 weeks elicited virtually identical yields of papillomas. The incidence of carcinomas on the other hand was less than maximal for mice promoted for 24 weeks or longer. In fact, Hennings et al. (1985) have shown that papillomas induced by the first few TPA treatments are much more likely to progress to carcinomas than those which appear later. It was of interest to determine the effects of promotion duration on the incidence of papillomas, carcinomas and the malignant conversion rate in mice initiated with ionizing radiation.

An examination of the proliferative state of the skin prior to irradiation and promotion duration after irradiation will give one a better understanding of mechanisms involved in initiation by ionizing radiation. In this chapter, experiments involving the induction of squamous and basal cell carcinomas in mice initiated with ionizing radiation will be described along with how the proliferative state of the skin and promotion duration effect the tumor incidence.

**Materials and Methods**

Female CD-1 mice, 5 to 7 weeks of age, were used for these studies. The animals were purchased from Charles River, Boston, MA, USA. Five animals each were housed in stainless-steel cages in light- and temperature-controlled rooms. Food and water were available ad libitum. The dorsal skin of the mice was shaved 3 to 4
days prior to treatment, and only those animals in the resting phase of the hair cycle were used for experimentation.

TPA (purchased from Chemicals for Cancer Research, Chanhassen, MN) solutions were prepared in acetone and were administered to shaved backs of animals in a volume of 0.2 ml. Control mice were treated with the same volume of acetone. Animals that were pretreated with TPA were painted with 17 nmole 24 hours prior to irradiation. TPA promotion was started 2 weeks after irradiation. To study the consequence of promotion duration on total tumor incidence, animals were treated twice per week with 8 nmole TPA for either 10 or 60 weeks. Acetone treatment was started in week 11 for the group of animals treated for 10 weeks with TPA and continued for the remainder of the study.

The mice were X-irradiated using a 4 MeV linear accelerator at a dose rate of 3.1 Gy per minute as previously described (Chapter 2). Briefly, a single fraction dose was delivered to an unopposed 2 x 8 cm field of dorsal skin. Animals were irradiated with a total dose of either 0.5 or 11.25 Gy. To examine the influence of target cell proliferation on tumor incidence, one half of the animals in the study were pretreated with TPA prior to irradiation. The remaining half were pretreated with acetone as a control. Animals were promoted as described above. The control group of animals were treated with acetone alone.

All groups contained between 20 and 30 animals. During the entire period of the study, cages were checked twice per week for
dead or moribund animals. Once per week tumors were counted, measured and the location noted. It was possible to follow individual tumors over the course of the study and to visualize their progression due to the low incidence of tumors per animal. Necropsies were performed at the time of termination. Tumor sections were fixed in 10 percent phosphate-buffered formalin. Histopathological evaluations were made by Dr. J.N. Shively, Department of Veterinary Sciences, University of Arizona.

To investigate whether tumor incidence rates differed between treatment groups we used the X² test (Snedcor and Cochran, 1980). Time to first tumor was compared between treatment groups using the log rank test (Peto et al., 1976; Peto et al., 1977) as per the method of Gail et al. (1980). Statistical analyses were performed by Dr. Dalice Sim, Director, Clinical Trials Resources Group, John P. Robarts Research Institute, London, Ontario.

Results

The results obtained from experiments involving the use of ionizing radiation as an initiator followed by various TPA promotion regimes are depicted in Figure 4. The overall tumor incidence observed with animals that were irradiated with either 0.5 or 11.25 Gy followed by 60 weeks of TPA promotion (0.36 and 0.44 tumors per mouse, respectively) was not significantly different from each other as measured by the X² test (p > 0.05). This was also true for animals that were promoted with TPA for only ten weeks (0.09 vs 0.08
tumors per mouse). Thus, no significant ($\chi^2 p > 0.05$) dose effect between groups of animals irradiated with 0.5 and 11.25 Gy was observed, provided that the animals received the identical promotion regime. Animals that were not irradiated had a significantly lower tumor incidence ($\chi^2 p < 0.01$) than their irradiated counterpart. Figure 4 also shows that the length of TPA promotion significantly increased the total tumor incidence. However, as indicated in Table 6 this was primarily due to an increase in the incidence of papillomas and was found to be independent of radiation dose.

Although there was not a significant difference in the total tumor incidence between groups of animals irradiated with 0.5 and 11.25 Gy, distinct differences in tumor type were observed as shown in Table 6. Animals irradiated with 0.5 Gy and treated with acetone alone did not develop any tumors over the entire period of the study. A 14 percent incidence in basal cell carcinomas was seen in animals that were irradiated with 11.25 Gy followed by acetone treatments. As mentioned briefly above, all animals that were promoted with TPA developed papillomas. The incidence of papillomas was dependent on the length of TPA promotion and independent of radiation treatment. Unlike what is usually seen with chemical initiators, regression of papillomas did not occur regardless of the length of TPA promotion. In fact animals promoted for only 10 weeks developed papillomas after cessation of promotion.

Squamous cell carcinomas were only observed in animals that had been irradiated and promoted. All squamous cell carcinomas were
Figure 4. Time Dependent Tumor Yield in CD-1 Mice After a Subcarcinogenic Dose of Radiation.

Animals were x-irradiated using a 4 MeV linear accelerator. A single dose of 0.5 or 11.25 Gy was delivered to a 2 x 8 cm field of dorsal skin. Control animals were not irradiated. Two weeks after irradiation, promotion was started. Animals were either treated with 0.2 ml acetone 2 times per week for 60 weeks (A); promoted with 8 nmol TPA n 0.2 ml acetone 2 times per week followed by 50 weeks of acetone treatment (O); or promoted for a total of 60 weeks (E).
Table 6.
Tumor Incidence in CD-1 Mice Initiated with Ionizing Radiation and Promoted with TPA

<table>
<thead>
<tr>
<th>Treatment$^a$</th>
<th>X-ray (Gy)</th>
<th>Promotion (week)</th>
<th>% Animals with Tumor</th>
<th>Tumors per Mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total $^b$</td>
<td>Papilloma</td>
<td>Squamous</td>
<td>Basal</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>Papilloma</td>
<td>Squamous</td>
<td>Basal</td>
</tr>
<tr>
<td>0.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>(0/25)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>10</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>(1/23)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>60</td>
<td>32</td>
<td>23</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>(7/22)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11.25</td>
<td>0</td>
<td>14</td>
<td>0</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>(3/22)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11.25</td>
<td>10</td>
<td>8</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>(2/25)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11.25</td>
<td>60</td>
<td>44</td>
<td>32</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>(11/25)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.0</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>(0/20)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.0</td>
<td>60</td>
<td>28</td>
<td>22</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>(6/23)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Treatment sequences are specified under Materials and Methods

$^b$ Numbers in parentheses represent the tumor-bearing animals divided by the total animal number.
observed to arise from pre-existing papillomas with an average latency time of 16 to 22 weeks following the development of a papilloma. Animals irradiated with 11.25 Gy and promoted with TPA for 10 to 60 weeks developed squamous carcinomas with the same frequency. However, the papilloma incidence in these two groups was significantly different. Animals that were irradiated with 0.5 Gy and promoted for only 10 weeks did not develop squamous cell carcinomas. This indicated that a larger total dose of TPA was required to induce the formation of squamous cell carcinomas at the lower dose of radiation. The incidence of squamous cell carcinomas was virtually identical in groups of animals irradiated with 0.5 Gy and promoted for 60 weeks and those animals irradiated with 11.25 Gy and promoted for 10 or 60 weeks (Table 6).

In Figure 5, the effect of TPA pretreatment on the initiating potential of ionizing radiation is illustrated. The overall tumor incidence in animals irradiated with 0.5 or 11.25 Gy followed by 60 weeks of promotion was 0.5 and 0.44 tumors per mouse, respectively. These values were not significantly different as measured by the X² test, p > 0.05. There was also no significant dose effect between any group of animals receiving the same promotion protocol. In Table 7, the influence of TPA pretreatment on animals irradiated with 0.5 or 11.25 Gy and promoted for 60 weeks are presented in more detail. As shown previously (Tables 3, 4, 6), all animals irradiated and promoted for 60 weeks develop squamous cell carcinomas. The highest incidence of squamous cell carcinomas occurred in animals
that were pretreated and irradiated with 0.5 Gy (13 percent). The lowest incidence of squamous cell carcinomas was found in the group of animals pretreated and irradiated with 11.25 Gy (4 percent). TPA pretreatment did not significantly alter the incidence of squamous cell carcinomas at either dose of radiation. Animals irradiated with 0.5 Gy and promoted for 10 weeks did not develop squamous cell carcinomas regardless of TPA pretreatment. TPA pretreatment did not have any effect on latency time or tumor incidence in CD-1 mice.

As shown in Table 8 the conversion rate of papillomas to squamous cell carcinomas varies from 15 to 75 percent depending on treatment modality. Squamous cell carcinomas were observed in all groups of animals that were irradiated and promoted with TPA for 60 weeks regardless of radiation dose. However, only animals irradiated with 11.25 Gy developed squamous carcinomas when promoted for only 10 weeks. Although the malignant conversion rates are very different between animals irradiated with 11.25 Gy and promoted for 10 vs 60 weeks, (75 vs 15 percent), the actual cumulative number of carcinomas in these two groups is identical. The cumulative number of papillomas, however, varies by a factor of 5.

In Table 9 animals were grouped according to the initiating dose of ionizing radiation they received regardless of TPA pretreatment and promotion duration. The data presented in this table shows a dose-dependent increase in the incidence of basal cell carcinomas: 0.7 percent for animals irradiated with 0.5 Gy and 4.2
Figure 5. Effect of TPA Pretreatment on Time Dependent Tumor Yield in CD-1 Mice After a Subcarcinogenic Dose of X-rays.

Animals were treated as described in Figure 4 legend except they were all treated with 17 nmol TPA in 0.2 ml acetone 24 hours prior to irradiation. Two weeks after irradiation, animals were either treated with 0.2 ml acetone 2 times per week for 60 weeks (A); promoted with 8 nmole TPA in 0.2 ml acetone 2 times per week for 20 weeks followed by 50 weeks of acetone treatment (O); or promoted with TPA for a total of 60 weeks (□).
### Table 7.
Effect of TPA Pretreatment on Tumor Incidence in CD-1 Mice Initiated with Ionizing Radiation and Promoted with TPA

<table>
<thead>
<tr>
<th>Pre-treatment</th>
<th>X-ray (Gy)</th>
<th>Promotion (week)</th>
<th>% Animal with Tumor</th>
<th>Tumors per Mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total b Papilloma Squamous Basal</td>
<td></td>
<td></td>
<td>Total Papilloma Squamous Basal</td>
</tr>
<tr>
<td>TPA</td>
<td>0.5</td>
<td>60</td>
<td>38</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>(9/24)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>--</td>
<td>0.5</td>
<td>60</td>
<td>32</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>(7/22)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TPA</td>
<td>11.25</td>
<td>60</td>
<td>28</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>(7/25)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>--</td>
<td>11.25</td>
<td>60</td>
<td>44</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>(11/25)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Treatment sequences are specified under Materials and Methods.

b Numbers in parentheses represent the tumor-bearing animals divided by the total animal number.
percent for animals irradiated with 11.25 Gy. The incidence of basal cell carcinomas was independent of promotion time (Table 6 and data not shown). The incidence of squamous cell carcinomas, however, did not appear to depend on dose. Animals treated with 0.5 Gy had a squamous cell carcinoma incidence of 5.3 versus 5.9 percent for animals irradiated with 11.25 Gy. However, since it is not known which portion of the dose response curve these two doses reside on, one cannot accurately say that the incidence of squamous cell carcinomas was not dose-dependent. A wider range of doses of ionizing radiation needs to be studied.

**Discussion**

Hulse (1967) has shown that the efficiency with which ionizing radiation acts as a complete carcinogen in mouse skin is dose-dependent. The peak yield of Epidermal tumors occurred at 21 Gy. In this chapter, data is presented which show that ionizing radiation can act as an initiator in mouse skin and that a single dose of 0.5 Gy can induce malignant epidermal tumors if followed by 60 weeks of TPA promotion (Table 6). Using ionizing radiation as an initiator, the incidence of squamous cell carcinomas was observed to be dependent on the dose of the initiator and the duration of promotion. Given a long enough period of promotion the incidence of squamous cell carcinomas was virtually identical at 0.5 and 11.25 Gy. However, at a higher dose of initiation (11.225 Gy) a shorter period of promotion with TPA is required (10 weeks) to result in
Table 8.
Conversion of Papillomas to Squamous Cell Carcinoma

<table>
<thead>
<tr>
<th>Dose (GY)</th>
<th>Promotion (WK)</th>
<th>% Conversion</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.5</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>0.5</td>
<td>60</td>
<td>25 (5/20)*</td>
</tr>
<tr>
<td>11.25</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>11.25</td>
<td>10</td>
<td>75 (3/4)</td>
</tr>
<tr>
<td>11.25</td>
<td>60</td>
<td>15 (3/20)</td>
</tr>
</tbody>
</table>

*Cumulative number of carcinomas divided by cumulative number of papillomas.
Table 9.
Carcinoma Incidence in CD-1 Mice Initiated With Ionizing Radiation

<table>
<thead>
<tr>
<th>Dose (GY)</th>
<th>% Animals with Carcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal</td>
</tr>
<tr>
<td>0.5</td>
<td>0.7 (1/144)*</td>
</tr>
<tr>
<td>11.25</td>
<td>4.2 (6/148)</td>
</tr>
</tbody>
</table>

*Number in parentheses represents the tumor bearing animals divided by the total animal number.
development of squamous cell carcinomas. In this group the squamous cell carcinoma incidence was similar to those animals that were irradiated followed by 60 weeks of TPA treatment (Table 6).

The data, presented in Chapter 2, indicate that in Sen car mice TPA pretreatment led to a significant increase in tumor incidence without an apparent effect on induction time. In the present study TPA pretreatment also did not affect the latency time. Actually, TPA pretreatment only slightly influenced the incidence of squamous cell carcinomas (Table 7). The highest incidence of squamous carcinomas occurred in animals that were pretreated, irradiated with 0.5 Gy and promoted for 60 weeks (13 percent). The lowest incidence was seen in animals that were pretreated, irradiated with 11.25 Gy and promoted for 60 weeks (4 percent). However, the effect of TPA pretreatment on the incidence of squamous carcinomas was not significant. These data suggest a possible enhancement at low doses and a toxic effect at high doses X-rays. Burns and colleagues (Albert et al., 1969; Vanderlaan, Burns and Albert, 1976; Burns et al., 1978a; Burns and Albert, 1986) have extensively studied rat skin tumors induced by carcinogenic doses of ionizing radiation. They have stimulated rat skin cell proliferation by plucking hair repeatedly or stripping the skin surface with cellophane tape repeatedly prior to irradiation and found no difference in tumor yield (Burns and Albert, 1986). It is possible that the changes observed in Sen car (Chapter 2) and the differences seen in CD-1 mice (Table 7, Figure 5) pretreated with TPA prior to
irradiation are not the result of TPA induced cellular proliferation but rather some other function of TPA. It will be of interest to examine the effect of pretreatment with non-promoting hyperplastic agents and further examine the observed strain differences.

Epidermal papillomas are the most frequently observed type of lesion produced in mouse skin as a result of the initiation-promotion protocol. This has been shown for many different chemical initiators (reviewed in: Yuspa, Hennings and Saffiotti, 1976; Scribner and Suss, 1978) as well as for initiating doses of UV and ionizing radiation (Shubik, et al., 1953; Stenback, 1975; Strickland, 1982; Chapter 2). Burns et al. (1978b) have shown that greater than 90 percent of epidermal carcinomas develop from pre-existing epidermal papillomas. However, the conversion of papillomas to the malignant phenotype is only 7 to 10 percent using chemical initiators. It was of interest to find a malignant conversion rate between 33 and 45 percent in the previous study using Sencar mice and ionizing radiation as an initiator (Chapter 2). In the present study, a malignant conversion rate of 15 to 75 percent depending on treatment modality was observed (Table 8). Although the malignant conversion rate was very different between animals irradiated with 11.25 Gy and promoted for 10 or 60 weeks (75 vrs 15 percent), the actual cumulative number of carcinomas in these two groups was identical. The cumulative number of papillomas, however, was quite different (Table 8). Using chemical initiators in this model system, it has been shown that papillomas induced by
the first TPA treatments are more likely to progress to carcinomas than those that appear later (Hennings et al., 1985). The results presented here are in agreement with these published results and support the contention that conditional papillomas contribute very little to the carcinoma yield. Thus, it would appear from these results and those presented in the literature for chemical initiators that the proportion of conditional versus autonomous papillomas is independent of the type of initiator, provided that TPA is used as the promoting agent.

At a dose of 0.5 Gy there appears to be a correlation between promotion duration and the carcinoma incidence. This is not the case for animals irradiated with 11.25 Gy. At this dose the longer promotion period resulted only in a higher incidence of papillomas. It will be of interest to examine promotion times less than 10 weeks. The data presented in both Chapters 2 and 3 indicate that the incidence of squamous carcinomas requires irradiation followed by TPA promotion. However, the amount of TPA promotion actually required for malignant tumor formation remains to be determined.

This is one of the first reports of basal cell carcinomas occurring in mice exposed to the initiation-promotion regime (Chapter 2). Basal cell carcinomas occur rarely in experimental animals. The exception is rat skin exposed to carcinogenic doses of ionizing radiation (Albert et al., 1969). In these experiments the incidence of basal cell carcinomas was dependent on the dose of ionizing radiation and independent of TPA promotion. This is in
contrast to squamous cell carcinomas whose incidence is dependent on the interaction of ionizing radiation and TPA. Basal cell carcinomas are cutaneous epidermal tumors characterized by nests or sheets of small basal-type cells having relatively large oval basophilic nuclei. They are a slow growing tumor and are the most abundant form of skin cancer found in humans (Domonkos, Arnold and Odom, 1982; Sanders and Kathren, 1983). Squamous cell carcinomas on the other hand are more rapidly growing tumors consisting of epidermal keratinocytes characterized by invasive nests of cells showing variable central keratinization (Domonkos, et al., 1982; Sanders and Kathren, 1983). The mechanism of induction of these two malignant tumor types and how ionizing radiation interacts with the skin to induce these lesions remains unknown. A more fundamental question to address is the nature of the target cells for these two lesions. It is known that 10 percent of the cells in the basal layer of mouse skin are clonigenic. These stem cells will divide and the daughter cells which are "programmed" to differentiate will divide 2 additional times before their daughters migrate into the spinous layer (Potten, 1985). Are cells that have been "programmed" to differentiate, but still reside in the basal layer, the target cells involved in the induction of squamous carcinomas? Are the more immature stem cells targets for basal carcinomas? Is it possible that the target cells for these two types of cancer are the same and it is the way the cell interacts with a carcinogen that determines the differentiation state of the
tumor? These are very interesting questions that need to be examined.

The results presented here confirm earlier findings that ionizing radiation is a weak initiator in mouse skin. These data indicate that the interaction between ionizing radiation and TPA in vivo, analyzed in terms of tumor response, is complex. The final outcome is dependent on the total dose of irradiation delivered to the dorsum, length of promotion and the target cells involved in the tumor response.
CHAPTER 4

IONIZING RADIATION ENHANCES MALIGNANT PROGRESSION
OF MOUSE SKIN TUMORS

In several experimental systems the multistage process of
carcinogenesis can be divided into initiation, promotion,
progression and metastasis. In mouse skin, operational distinction
between initiation and promotion has been clearly demonstrated.
Initiation has been defined as a single subthreshold dose of a
carcinogen and evidence indicates that it involves the induction of
genetic alterations that are essentially irreversible. Promotion
involves repetitive applications of a non-carcinogenic agent.
While, promotion may result in the clonal expansion of initiated
cells it may also facilitate the development of certain neoplastic
characteristics in initiated cells. The initiation-promotion
protocol in mouse skin produces predominantly papillomas and, to a
much lesser degree, squamous cell carcinomas. Progression is the
process by which these benign papillomas develop malignant
characteristics and is believed to involve a number of genetic
alterations (Slaga, 1972). Metastasis of squamous cell carcinomas
of mouse skin rarely occur using the standard protocol and are
thought to require further genetic changes.
Carcinoma development in the mouse skin two-stage tumorigenesis protocol using chemical initiators is usually a relatively rare and late event. The frequency is dependent on mouse strain initiator and promotion protocol. In general approximately 10 percent of papillomas progress to squamous carcinomas and until quite recently very little has been reported on the modification of malignant progression. Recently, several investigators have treated mice that have skin papillomas with tumor initiators and have observed an increase in the carcinoma formation compared to control mice treated with the tumor promoter TPA (Hennings et al., 1983; O'Connell et al., 1986a, 1986b). These data suggest that progression to malignancy is the result of genetic changes caused by mutagenic agents.

When benzoyl peroxide, a free radical generating agent, was used as a tumor promoter in the two-stage protocol, it was found to be a moderately active promoter. However, unlike the classical tumor promoter TPA, benzoyl peroxide caused an unexpectedly high ratio of carcinomas relative to papillomas (Reiners et al., 1984). Recently, O'Connell et al. (1986b) have treated papilloma bearing mice with benzoyl peroxide and have shown that in addition to its promotional activity, benzoyl peroxide is highly active in malignant progression.

Data presented in Chapters 2 and 3 demonstrate that ionizing radiation acts as a weak initiator in the mouse two-stage skin
model. An unexpectedly high percentage of papillomas progressing to squamous carcinomas was observed in these studies. The experiment presented in this chapter was designed to test the hypothesis that ionizing radiation may be highly active in malignant progression, the third stage of carcinogenesis. This chapter shows that ionizing radiation has the ability to alter malignant progression.

Skin tumors were chemically induced using a standard two-stage initiation promotion protocol in female CD-1 mice (Charles River, Boston, MA, USA). Initiation was accomplished by a single topical application of 5 μmol N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) on shaved dorsal skin. Biweekly promotion with 8 nmol TPA in 0.2 ml acetone was begun 2 weeks after initiation. Control animals were treated with acetone alone. After 20 weeks of promotion, the papilloma yield had stabilized. The animals were then treated with either acetone, TPA (2 times per week for 2 weeks) or 8 fractions of 1 MeV electrons (1 Gy per fraction, 4 days on, 2 days off, 4 days on). Superficial radiotherapy treatments to the dorsal surface of the mice were administered using a Clinac-18 linear accelerator in the 6 MeV electron beam mode. A special jig (Figure 6) was fabricated which immobilized up to 14 mice in separate compartments and was covered with a 6.23 mm thick lucite lid in contact with the dorsal surface of the mice. An appropriately shaped 3 mm thick lead shield was used to collimate the 29 x 29 cm open electron field to 2 x 8 cm areas centered over each mouse.

The 6 MeV electron beam was degraded to an energy of 1 MeV
(practical range, $R_p = 0.41 \text{ cm}$) at the target surface by placing a composite lucite-polystyrene decelerator (thickness $2.85 \text{ g/cm}^2$) on top of the jig. Depth dose measurements in a polystyrene phantom using a Capintes parallel-plate chamber indicated that the maximum dose occurred at the skin surface and the 80%, 50% and 20% depth doses occurred at depths of 0.8 mm, 2.2 mm and 4.0 mm, respectively. Using film dosimetry and additional ionometric measurement, the dose distribution was found to be uniform within 10% over 80% of the area defined by the 14 apertures. Absolute calibration of the set-up (cGy at skin surface/monitor unit) was established in accordance with the calibration protocol recommended by Task Group 21 of the American Association of Physicists in Medicine (Lempert, Nath and Schultz, 1983).

All groups contained 20 animals. Once per week tumors were counted and measured and the location was noted. Necropsies were performed at the time of termination. Tumor sections were fixed in 10% phosphate-buffered formalin. Histopathological evaluations were made by Dr. J.N. Shively, Department of Veterinary Sciences, University of Arizona. To investigate the difference in tumor yield between groups, the Fisher's Exact Test (Rosner, 1986) was used.

Animals that received stage I MNNG plus stage II acetone or stage I acetone plus stage II acetone or TPA all had a similar incidence of papillomas at the time of stage III irradiation. The tumor incidence in these groups of animals was very low and was not altered by stage III treatment.
Figure 6. Schematic of Irradiation Protocol.

Animals were injected with nembutal (50 mg/kg) and placed in the jig. A 6.23 mm thick lucite lid was used to cover the animals and a lead shield (3 mm thick) was used to collimate the 29 x 29 cm open electron field to 2 x 8 cm areas centered over each mouse. The 6 MeV electron beam was degraded to an energy of 1 MeV at the target surface as described in the text. The animals received 1 Gy for 4 consecutive days followed by 2 days without radiation treatments and 4 more days of consecutive treatments. The animals received a total dose of 8 Gy given in 8 fractions over a 10 day period.
The overall tumor incidence between groups of animals initiated with MNNG, promoted with TPA for 20 weeks and then either irradiated or treated for two additional weeks with TPA was not significantly different as measured by the Fisher's Exact Test, $p > 0.05$ (Figure 7A). However, there was a large difference in the development of carcinomas between these two groups (Figure 7B). With TPA treatment in stage III approximately 25 percent of the papillomas progressed to carcinomas. Carcinomas occurred between weeks 35 and 40. The rate of malignant conversion for stage III treatment with ionizing radiation reached 80 percent by week 45. Carcinomas occurred earlier (after 25 to 30 weeks) when ionizing radiation was used for stage III. Once the papilloma yield had stabilized there was no increase in the cumulative papillomas per group during the progression stage. These results indicate that the production of carcinomas was due to an effect of ionizing radiation on pre-existing papillomas and not the result of development of new papillomas with a high tendency to become carcinomas.

In all groups of animals with papillomas, regardless of stage I and stage II treatment, there was a regression in the size of papillomas following stage III radiation. This regression was first observed two weeks following irradiation and continued for an additional four weeks. Papillomas seldom regressed fully. Regrowth appeared to occur from the base of the papilloma and the classical stalk structure never reappeared. Histopathological examination was performed on all tumors and the only carcinomas to develop from
Figure 7. Time Dependent Tumor Incidence and Rate of Malignant Conversion in CD-1 Animals.

Time Dependent Tumor Incidence in CD-1 Animals (A). Comparison of the Rate of Malignant Conversion in Groups of Papilloma-bearing Mice Treated in Stage III with Either TPA or Ionizing Radiation (XRT) (B). Animals were initiated with 5 μmol MNNG and promoted for 20 weeks with TPA (8 nmol 2 times per week). Animals were then treated with either acetone (Δ); TPA (○); or XRT ([]) as described in Chapter 4.
stage III radiation occurred in animals receiving MNNG stage I and TPA stage II. This could merely be the result of tumor number since animals not treated with the combination of MNNG initiation and TPA promotion had a much lower incidence of papillomas at the time of stage III irradiation. The increase in malignant conversion rate following stage III irradiation may be the result of specific interactions between the cells with neoplastic potential and ionizing radiation. Although there was a difference in malignant conversion rates between groups of animals initiated with MNNG, promoted with TPA and then either irradiated or treated with TPA for two additional weeks, the difference was not significant (Fisher's Exact Two-Tailed test, p=0.21). This lack of significance might be due to small sample size (4 carcinomas per 5 papillomas vs 1 carcinoma per 4 papillomas respectively). To address this question further these studies have been redesigned in an attempt to increase the papilloma incidence at the time of stage III radiation in all groups of animals. Preliminary results from this ongoing study (using Sencar animals and DMBA initiation) also show an increase in malignant conversion rate following stage III irradiation, giving support to the findings presented here.

The mechanism by which ionizing radiation enhances malignant progression is currently unknown. As clearly shown, ionizing radiation does not have any promoting activity; no new papillomas developed after ionizing radiation in animals receiving stage I MNNG and stage II acetone. The data presented in Chapters 2 and 3
indicate that single fraction doses of ionizing radiation can act as a weak initiator in mouse skin. Whether initiation occurs with the fractionated dose regime is not yet clear. It is of interest to note that O'Connell et al. (1986b) have recently shown that peroxides (both benzoyl and hydrogen peroxide) can enhance tumor progression. Peroxides, like ionizing radiation, are capable of generating free radicals. These data are consistent with the hypothesis that free radicals play a role in the progression process.
CHAPTER 5

DETECTION AND CHARACTERIZATION OF DOMINANT TRANSFORMING GENES
IN RADIATION-INDUCED MOUSE SKIN TUMORS

Introduction

Approximately 10-25 percent of all human tumors and tumor-derived cell lines which have been tested contain DNAs with transforming activity as measured by the NIH3T3 focus assay (reviewed in: Cooper, 1982; Bishop, 1983; Land et al., 1983a). No correlations between NIH3T3 transforming activity, tumor histology, degree of malignancy or tissue specificity have been found with human tissue. A striking difference is observed among experimental animal tumors. The majority of tumors (60-90%) produced in mice and rats by defined carcinogenic protocols exhibit transforming activity in the NIH3T3 assay (Balmain and Pragnell, 1983; Eva and Aaronson, 1983; Sukumar et al., 1983; Guerrero et al., 1984a, 1984b; Bizub et al., 1986; Weisman et al., 1986; Sawey et al., 1987). Although most carcinogen-induced mutations detected in the NIH3T3 assay are mutated members of the ras gene family, other oncogenes not related to ras have been found to be active in this assay. These include met (Cooper et al., 1984), neu (Schecter et al., 1984), raf (Fukui et al., 1985), erbB (Fung et al., 1983), B-lym (Diamond et al., 1983) and dbl (Eva and Aaronson, 1985). There are also a number of
reports in the literature which provide evidence for dominant transforming activity in a wide range of animal tumors (induced by different carcinogenic protocols) that do not appear to be associated with the ras family of oncogenes or any of the genes listed above (McMahon et al., 1984; Fox and Watanabe, 1985; Garte et al., 1985; Pulciani et al., 1987). This indicates the presence of dominate transforming genes that have yet to be identified.

Ionizing radiation is a potent and well characterized carcinogen. As previously shown in Chapters 2 and 3, several types of tumors arise in mice initiated with ionizing radiation and promoted with TPA. The molecular mechanisms by which ionizing radiation results in tumorigenesis are not known. However, the reported genotoxic effects of this carcinogen include primarily rearrangements and translocations (Wust, Riggsby and Whitson, 1972; Graf and Chasin, 1982). A large number of radiation-induced rat skin and thyroid tumors have been shown to contain genomic amplification and/or rearrangements in the cellular myc gene (Sawey et al., 1987). In addition, ionizing radiation has also been demonstrated to result in activation of the cellular Ki-ras gene by somatic mutations in mouse lymphomas (Guerrero et al., 1984a, 1984b) and rat skin tumors (Sawey et al., 1987). In vitro malignant transformation of hamster and mouse C3H/10T 1/2 cells by x-rays results in activation of oncogenic sequences not related to the ras gene family or any of the oncogenes characterized so far (Borek et al., 1987). Oncogenes have been shown to be activated
primarily by point mutations (Reddy et al., 1982; Tabin et al., 1982; Taparowsky et al., 1982; Guerrero et al., 1984a, 1984b, 1984c; Zarbl et al., 1985; Quintanilla et al., 1986), gene amplification (Collins and Groudine, 1982; Alitalo, 1985), and/or chromosomal translocations (Crews et al., 1982; Dalla-Favera et al., 1982; deKlein et al., 1982; Harris et al., 1982; Neel et al., 1982; Rechavi et al., 1982; Shen-Ong et al., 1982; Adams et al., 1983; Marcu et al., 1983; Taub et al., 1984), all of which occur in ionizing radiation-induced malignant transformation. The mouse two-stage model of tumorigenesis, using ionizing radiation as an initiator offers an excellent system in which to study molecular mechanisms involved in radiation-induced cancer.

Results discussed in this chapter provide evidence for dominant transforming activity not associated with the ras family of oncogenes in radiation-induced mouse skin tumors. Data are also presented which demonstrate genomic amplification and rearrangement of c-myc in a few of the tumors examined.

**Materials and Methods**

**Nucleic Acid Extraction**

Nucleic acids were isolated according to the method of Krieg, Amtmann and Sauer (1983). Tumors were powdered in a mortar and pestle under liquid nitrogen. Cultured cells were trypsinized (0.05% Trypsin, 0.7 mM EDTA, 5.6 mM glucose, 5.4 mM KCl, 140 mM NaCl, 4.2 mM NaHCO₃, pH 7.2), pelleted and rinsed with an isotonic
buffer (10 mM EDTA, 130 mM NaCl, 50 mM Tris-HCl, pH 7.5). The ground tissue (0.5 g) and cultured cell pellets (4 x 10^7 cells) were lysed in a mixture of 5 ml phenol, 1% hydroxyquinoline equilibrated with 0.5 M NaAcetate pH 7.5, plus 2 ml 2 mM EDTA, 0.5% SDS, 0.3 M NaAcetate pH 7.5. This mixture was extracted twice with chloroform-isoamyl alcohol (24:1). The nucleic acids were precipitated in 2 volumes ice cold 100% ethanol. The ethanol-precipitated nucleic acids were pelleted by centrifugation and resuspended in sterile distilled H_2O. RNA was selectively precipitated by the addition of an equal volume of 4 M LiCl at 4°C for at least 4 hours. The precipitate was pelleted by centrifugation. The upper phase containing the DNA was removed and precipitated with 2 volumes ice cold 100% ethanol. DNA was washed in ice cold 70% ethanol, air dried and resuspended in 20 mM Tris-HCl, 1 mM EDTA, pH 7.5. RNA pellets were dissolved in sterile distilled H_2O, MgSO_4 and DNase I were then added to give a final concentration of 5 mM and 0.04 mg/ml, respectively. After a 30 minute room temperature incubation, the RNA solution was extracted once with an equal volume of phenol and twice with chloroform-isoamyl alcohol (24:1). One-tenth volume of 3 M NaAcetate was added to the aqueous phase and then it was precipitated with 2 volumes ice cold 100% ethanol. The RNA pellets were dried and dissolved in sterile distilled H_2O. The concentration and purity of the DNA and RNA were determined by spectrophotometric analysis at 260 and 280 nm.
Transfection Assays

Freshly propagated NIH3T3 or Rat-2 fibroblast lines were grown in Dulbecco's modified Eagle's medium, DMEM (Gibco), supplemented with 10% fetal calf serum (FCS) and antibiotics (100 units/ml each of penicillin and streptomycin). Transfection assays were performed by the calcium phosphate co-precipitation method of Wigler et al. (1979). Forty micrograms of DNA were dissolved in 2 ml of transfection mixture (0.5 mM Tris-HCl, 0.05 mM EDTA pH 8, 140 mM NaCl, 25 mM HEPES, 0.75 mM Na2HP04) and then precipitated with 0.1 ml 2.5 M CaCl2. After 30 minutes at room temperature, the transfection mixture was introduced into a 100 mm culture dish containing 2 x 10⁵ recipient cells and 3 ml of DMEM plus 10% FCS. Cells were incubated for 12-18 hours with the DNA precipitate and then freed from the DNA by extensive washing with DMEM plus 10% FCS (day 2). On day 4 the media was changed to DMEM plus 4% FCS. Thereafter, cells were fed with the same medium at intervals of three days. Three to four cultures were used per DNA sample. As controls, each transfection assay included four cultures treated with calcium phosphate alone (mocks), DNA from untreated NIH3T3 cells (negative control) and DNA from a human T₂₄ bladder carcinoma cell line (positive control; Reddy et al., 1982). Morphologically transformed foci were scored at 16-24 days. Foci were picked and transformed cells of interest were grown separately and used as sources of DNA for second and third transfection experiments.
Restriction Endonuclease Functional Mapping

Mapping was performed on selected primary transfectant DNA. The DNA were digested with a panel of restriction enzymes: Bam H1, EcoRI, Hind III and Pvu II (Amersham) according to the specifications provided by the supplier for each enzyme. Basically, DNA dissolved in 50mM NaCl, 10mM Tris-Cl (pH 7.5), 10 mM MgCl₂ and 1mM dithiothreitol was incubated at 37⁰C for 24 hours in the presence of 10 units restriction endonuclease per ug DNA. Aliquots of all digested DNAs were electrophoresed on 1% agarose gels to determine degree of digestion. DNAs that were digested to completion were used in secondary transfection experiments.

Growth in Soft Agar

Transformed cells were tested for anchorage independent growth by cloning in soft agar using the method of MacPherson and Montagnier (1964). A base layer of 0.5% agar, 1 x DMEM and 10% FCS was added to 60 mm plastic culture dishes. Diluted cell suspensions containing a final concentration of 0.33% agar were layered onto culture dishes containing the hardened agar medium. Three dilutions were used per cell line; three plates per dilution. Colonies were counted at week 2 and 3. Negative (NIH3T3) and positive (T₂₄; Reddy et al., 1982) control cell lines were run in each assay. Cloning efficiencies were expressed as a percentage of the number of colonies scored with respect to the number of cells seeded per plate.
Tumorigenicity in Nude Mice

Transfectant clones were tested for their ability to produce tumors in nude mice. Male (5-7 weeks) athymic nude mice were purchased from Harlan, Sprague Dawley, Walkersville, Maryland. Nude mice were injected with 1-2 x 10^6 cells in 0.2 ml DMEM without FCS, subcutaneously into the hind flanks. As controls, NIH3T3 (negative) and T24 (positive) cell lines were subcutaneously injected into mice as described above. Mice were housed in autoclaved cages and given autoclaved food and H2O ad libitum. All handling, injections and inspections were done in a laminar flow hood. Mice were examined weekly. Tumors that appeared were sectioned for histologic analysis. This analysis was performed by Dr. J. N. Shively, Department of Veterinary Sciences, University of Arizona. Tumors were immediately frozen in liquid nitrogen and stored at -80°C for future use.

Southern Analysis

DNA from tumors and transfectants were digested with restriction enzymes using the conditions recommended by the supplier for each enzyme. The digested DNAs (10 µg) were separated by electrophoresis through horizontal agarose slab gels and transferred to nitrocellulose filters (Southern, 1975). Nick-translated ^32P-labeled probes with a specific activity of 3-10 x 10^7 cpm/µg DNA were hybridized to the nitrocellulose filters in 50% formamide, 5 x Denhardt's, 5 x SSC, 0.1% SDS and 100 mg/ml denatured salmon sperm
DNA according to Maniatis et al. (1982) at 43°C for 18-24 hours. Filters were washed 3 times, for 10 minutes each, in 2.0 x SSC, 0.1% SDS at room temperature. The final wash stringency was 0.2 x SSC, 0.1% SDS at 55°C for B-lym, erbB, met, myc, neu and raf probes and 0.2 x SSC, 0.3% SDS at 65°C for Ha-, Ki, and N-ras probes. Autoradiography was performed with Kodak XAR film and intensifying screens.

Northern Analysis

Total RNAs (10 μg) were separated by horizontal slab gel electrophoresis in 1% agarose, 2.2 M formaldehyde, 1 x MOPS gels (Maniatis et al., 1982) and transferred to nitrocellulose as described above. Hybridization and wash conditions were identical to those used for Southern analysis.

RNA dot blot analysis was performed by dissolving 24 μg of total RNAs into 200 μl of 15 x SSC, 8 M formaldehyde. Samples were heat denatured and then serial dilutions were made in the identical buffer, 100 μl of each sample was spotted onto nitrocellulose. Hybridization conditions were the same as those listed above. The pA6 probe (7s) was used as a control for amount of RNA loaded.

Probes

The following probes were provided by the indicated scientists: 1) Allan Balmain, Beatson Institute for Cancer Research, Glasgow, psp659EB2-ras (mouse Ha-ras); H1H13 (viral Ki-ras, Ellis et al., 1982); pmyc-2 (viral myc, Vennstrom et al.,
1981); pA6 (7s, Balmain et al., 1982); 2) Angel Pellicer, New York University Medical center, New York, mouse N-ras exon 1 and 2; 3) Geoffrey Cooper, Harvard Medical School, Boston, pHuBlym (Diamond et al., 1983); 4) Robert Weinberg, Massachusetts Institute of Technology, Cambridge, pSV2-neu (Bargmann et al., 1986a, 1986b); 5) American Type Culture Collection, Rockville, Maryland, v-erbB, c-raf-1, pmet- H.

Plasmids were prepared according to the alkaline lysis method of Maniatis et al. (1982). Restriction fragments were isolated from indicated plasmids after resolution on low-melting-point agarose gels. Probes which were used in these studies are: pSp659EB2-ras, 600 base pair (bp) EcoRI/BamHI insert; HIHI3, 380 bp SstII/Xba I insert; mN-ras exon 1, 750 bp EcoRI/Hind III insert; mN-ras exon 2, 900 bp Pst I/Xba I insert; pmet-H, 1.6 Kbp SalI/EcoRI insert; pmyc-2, 1.5 Kbp Pst I insert; c-raf-1, 1.6 Kbp EcoRI/Hind III insert. Plasmids and inserts were radiolabeled with a $^{32}$P dCTP (3200 Ci/mmol) ICN using an Amersham nick translation kit (Rigby et al., 1977) and hybridized to nitrocellulose filters as described above.

**Results**

Detection of Dominant Transforming Genes

DNAs prepared from 20 radiation induced mouse skin tumors (Chapter 2 and 3) were examined for their ability to transform NIH3T3 cells (Table 10). High-molecular weight DNAs from both Sencar and CD-1 papillomas, squamous and basal cell carcinomas, and
### Table 10

Transforming Efficiency of Radiation Induced Tumors

<table>
<thead>
<tr>
<th>Source of DNA</th>
<th>Strain</th>
<th>Tumors Tested</th>
<th>Transformation Efficiency* (foci/µg DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>NIH3T3</td>
</tr>
<tr>
<td>T24</td>
<td>----</td>
<td>----</td>
<td>0.4</td>
</tr>
<tr>
<td>Pooled Papillomas</td>
<td>Sencar</td>
<td>3</td>
<td>0.02-0.08 (2/3)**</td>
</tr>
<tr>
<td></td>
<td>CD-1</td>
<td>1</td>
<td>0.07</td>
</tr>
<tr>
<td>Squamous Carcinomas</td>
<td>Sencar</td>
<td>5</td>
<td>0.05-0.3 (4/5)</td>
</tr>
<tr>
<td></td>
<td>CD-1</td>
<td>4</td>
<td>0.04-0.2 (4/4)</td>
</tr>
<tr>
<td>Basal Carcinomas</td>
<td>Sencar</td>
<td>3</td>
<td>0.03-0.13 (2/2)</td>
</tr>
<tr>
<td></td>
<td>CD-1</td>
<td>1</td>
<td>0.6</td>
</tr>
<tr>
<td>Adeno-carcinomas</td>
<td>Sencar</td>
<td>3</td>
<td>0.03-0.6 (3/3)</td>
</tr>
<tr>
<td></td>
<td>CD-1</td>
<td>1</td>
<td>0.2</td>
</tr>
</tbody>
</table>

*Transformation efficiency was calculated as foci/plate tumor DNA minus foci/plate mock transfection (usually 1-2 foci/plate) divided by [DNA]/plate.

** Number of positive samples/total number of samples tested.
adenocarcinomas were applied to NIH3T3 cells using the calcium phosphate co-precipitation technique. The NIH3T3 cells that were transformed by tumor DNAs were selected by scoring foci of morphologically transformed cells after 16-24 days. DNA from ninety percent (18/20) of all tumors tested transformed NIH3T3 cells. In control experiments, DNA from the T24 bladder carcinoma cell line efficiently induced foci while only very low levels of foci (< 0.01 foci/μg DNA) were observed when cells were transfected with DNA from normal mouse skin, liver and NIH3T3 cells. DNAs isolated from 12 NIH3T3 transfectants were used to transform Rat-2 cells. Of the 12 DNAs examined, 10 were able to transform Rat-2 cells. The two transfectant DNAs that did not transform Rat-2 cells were both derived from papillomas.

Probing for Activated Ras Genes

To determine if mouse DNA sequences were present in the Rat-2 foci, DNAs derived from the Rat-2 transfectants were examined for the presence of mouse sequences. Samples of EcoRI, Bam HI and Hind III digested DNAs were subjected to electrophoresis in agarose gels, transferred to nitrocellulose and probed for the presence of NIH3T3 sequences using 32P dCTP labeled NIH3T3 genomic DNA (data not shown). From these data, 5 cell lines were chosen for further analysis. Three cell lines, S1R/TSC1, S27SC7, and S212SC4 were derived from independent squamous cell carcinomas induced in Sencar mice (Chapter 2) by treating the animals with an initiating dose of
ionizing radiation followed by 60 weeks of TPA promotion. The cell lines C1R/TPPAP9 and C18SC13 were derived in a similar manner from CD-1 animals (Chapter 3). C1R/TPPAP originated from a group of pooled papillomas while C18SC13 was derived from a squamous cell carcinoma.

Probes prepared from the mouse Ha- and N-ras genes and Kirsten sarcoma virus oncogene were hybridized to Southern blots of primary NIH3T3 transfectant DNAs (Figure 8A). For all three ras genes, only the endogenous bands were present. There was no evidence for amplification and/or rearrangements in any of the transfectants examined. The variation in band intensities observed in Figure 8A were the result of uneven loading of DNA samples onto the gels. Secondary Rat-2 transfectant DNAs (Figure 8b) were probed with the same three ras genes to determine if the Rat-2 transfectants contained any mouse ras sequences. As shown in Figure 8b, the Rat-2 transfectants only contained the endogenous rat ras sequences. These results indicate that the putative transforming gene(s) activated in these five cell lines are not members of the ras gene family.

Characterization of the Transformed Phenotype

The transformed phenotype of the transfectant cell lines was further confirmed by growth in soft agar and tumorigenicity in nude mice. As shown in Table 11, the percent cloning efficiency varied from $0.64 \pm 0.22$ for primary S1R/TSC1 cell line to $3.0 \pm 3.1$ for
Figure 8. Southern Blot Analysis of Primary NIH3T3 (8A) and Secondary Rat-2 (8B) Transfectants Hybridized to Ha-, Ki- or N-ras Probes.

DNAs were from: lane N,: NIH3T3; lane R, Rat-2; lane 1, S1R/TSC1; lane 2, S27SC7; lane 3, S212SC4; lane 4, BC1R/TPPAP; lane 5, C18SC13. The Ha-ras probe was a 600 bp EcoRI/Bam HI insert of pSp659EB2-ras. Primary transfectants were digested to completion with EcoRI and secondary transfectants were digested with Bam HI. The Ki-ras probe was a 1000 bp EcoRI insert of HiHi3 for the primary transfectants and a 380 bp SstII/XbaI insert of HiHi3 for the secondary transfectants. Both primary and secondary transfectants were digested to completion with Hind III. The N-ras probe was a 750 bp EcoRI/Hind III fragment of mouse exon 1 and a 900 bp Pst1/XbaI fragment of mouse exon 2. Both primary and secondary transfectants were digested to completion with EcoRI. Size markers were Hind III-digested λ-phage DNA.
Table 11
Characterization of NIH3T3 Transfectant Cell Lines in Terms of Anchorage Independent Growth and Tumorigenicity in Nude Mice

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Percent Cloning Efficiency in Agar</th>
<th>Tumorigenicity No. Tumors Per Injection Site</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1*</td>
<td>2*</td>
</tr>
<tr>
<td>NIH3T3</td>
<td>( &gt; 0.1 \pm 0.11 )</td>
<td>--- (^1)</td>
</tr>
<tr>
<td>NIH3T3 ( T_{24} )</td>
<td>13 ( \pm 3.7 )</td>
<td>4/6 (16) (^2)</td>
</tr>
<tr>
<td>C1R/TPPAP9</td>
<td>30 ( \pm 3.1 )</td>
<td>8/8 (7)</td>
</tr>
<tr>
<td>BC1R/TPPAP</td>
<td>0.21 ( \pm 0.09 )</td>
<td>2/6 (57)</td>
</tr>
<tr>
<td>C18SC13</td>
<td>0.34 ( \pm 0.15 ) 0.84 ( \pm 0.20 )</td>
<td>0/6 2/4 (49)</td>
</tr>
<tr>
<td>S1R/TSC1</td>
<td>0.64 ( \pm 0.22 ) 3.5 ( \pm 0.71 )</td>
<td>4/6 (55) 9/10 (33)</td>
</tr>
<tr>
<td>S27SC7</td>
<td>1.7 ( \pm 0.06 ) 3.0 ( \pm 0.62 )</td>
<td>2/6 (80) 4/6 (41)</td>
</tr>
<tr>
<td>S212SC4</td>
<td>1.2 ( \pm 0.12 ) 2.6 ( \pm 0.55 )</td>
<td>3/6 (42) 6/6 (42)</td>
</tr>
</tbody>
</table>

\(^1\)Experiments not performed.

\(^2\) Average latency period in days
CIR/TPPAP9 primary cell line. For a given cell line the percent cloning efficiency always increased in the secondary transfectant. The tumorigenicity data confirms this selection of a transformed phenotype. For a given cell line there was an increase in the number of tumors per injection site as well as a decrease in the latency period in secondary transfectants compared to their primary counterpart. These data support the contention that the transforming activity was serially passaged during subsequent rounds of transfection. DNA from the most tumorigenic cell line CIR/TPPAP9 (based on growth in soft agar and tumorigenicity in nude mice) failed to transform NIH3T3 cells in secondary rounds of transfection. An independent primary focus from a CIR/TPPAP transfection experiment (BCIR/TPPAP) did not grow well in soft agar and was weakly tumorigenic in nude mice (2/6 tumors per injection site, average latency period 57 days). These and other data presented later indicate that the CIR/TPPAP9 cell line acquired its transforming activity during the transfection process and the acquisition of this activity was unrelated to events that had occurred in the corresponding tumor DNA.

The transforming gene(s) transferred by DNA from the four squamous cell carcinomas were further characterized by determining their sensitivity to digestion with a series of restriction enzymes. Digestion of tumor-derived DNA with a particular restriction endonuclease will inactivate transforming activity only if those sequences necessary for transformation contain a recognition site
for that specific restriction endonuclease. Digestion of a series of transforming DNAs with a number of different restriction endonucleases therefore allows a comparison of the restriction sites contained within the transforming sequences activated in different neoplasms. Data from the mapping experiments are summarized in Table 12. The transforming gene(s) of the transfectant cell lines S1R/TSC1 and S27SC7 was inactivated by HIND III, while EcoRI destroyed the transforming activity of S212SC4. C18SC13 was inactivated by the restriction enzyme BamHI. PvuII did not affect the activity of any of the four cell lines. These results suggest that the transforming activity in 2 out of 4 squamous cell carcinomas may be the same (S1R/TSC1 and S27SC7), and that there are possibly three separate transforming genes.

Screening Transfectants for the Presence of Known Cellular Oncogenes

The majority of oncogenes detected in the NIH3T3 focus assay have been shown to be genetically altered versions of 1 of 3 closely related ras genes. However, oncogenes unrelated to ras (B-lym, erbB, met, neu, raf,) have also been detected by DNA transfection assays. Southern blots of primary and secondary NIH3T3 transfectants were screened for the presence of amplified or rearranged B-lym (data not shown), erbB (Figure 9), met (data not shown), neu (Figure 10) and raf (Figure 11). These experiments revealed only endogenous mouse fragments. Amplification and rearrangements were not detected with any of the probes examined, in
<table>
<thead>
<tr>
<th>Donor DNA</th>
<th>Undigested</th>
<th>BamHI</th>
<th>EcoRI</th>
<th>HindIII</th>
<th>PvuII</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hs242</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>T24</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>S1R/TSC1</td>
<td>+</td>
<td></td>
<td>+*</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>S27SC7</td>
<td>+</td>
<td></td>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>S212SC4</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C18SC13</td>
<td>+</td>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*Results from repeated experiments were not consistent. In some cases, there was no inactivation of the transforming activity (+), while in repeat experiments, the transforming activity was inactivated (−).
Figure 9. Southern Blot Analysis of Transfectant DNAs Digested with XbaI and Hybridized to a Viral erbB Probe.

Primary NIH3T3 transfectant cell lines are shown in lanes 1-5, secondary NIH3T3 transfectant cell lines are shown in lanes 6-9. DNAs were from: lane N, NIH3T3; lanes 1 and 6, S1R/TSC1; lanes 2 and 7, S27SC7; lanes 3 and 8, S212SC4; lanes 4 and 9, C18SC13; lane 5, C1R/TPPAP9. Size markers were Hind III-digested λ-phage DNA.
Figure 10. Southern Blot Analysis of Primary and Secondary NIH3T3 Transfectant DNAs Digested with BamHI/EcoRI and Hybridized to a Human Neu Gene (psv2-neu).

Primary transfectant cell lines are shown in lanes 1-5 and secondary transfectant cell lines are in lanes 6-9. DNAs are from: lane N, NIH3T3; lanes 1 and 6, S1R/TSC1; lanes 2 and 7, S27SC7; lanes 3 and 8, S212SC4; lanes 4 and 9, C18SC13; lane 5, C1R/TPPAP9. Size markers were Hind III-digested λ-phage DNA.
Figure 11. Southern Blot Analysis of NIH3T3 Transfectant DNAs
Digested with PstI and Hybridized to a 1.6 Kbp
EcoRI/HindIII Insert of Human c-raf-1.

Primary transfectants are in lanes 1-5 and secondary transfectants
are shown in lanes 6-9. DNAs are from: lane N, NIH3T3; lanes 1 and
6, S1R/TSC1; lanes 2 and 7, S27SC7; lanes 3 and 8, S212SC4; lanes 4
and 9, C18SC13; lane 5, C1R/TPPAP9. Size markers were Hind III-
digested λ-phage DNA.
any of the transfectant cell lines studied. The exception was the cell line C1R/TPPAP9. As indicated earlier, this cell line was derived from a group of pooled papillomas isolated from CD-1 animals. As shown in Table 11, this cell line was highly tumorigenic, producing tumors at all injection sites within one week. In Figure 10 an extra band was observed in this cell line when probed with the neu oncogene (Figure 10, lane 5). DNA from an independent primary C1R/TPPAP focus did not reveal the extra neu band (data not shown). Experiments (data not shown) have demonstrated that this band was not present in the original pooled tumor DNA. Taken together with data presented in Table 11, evidence suggests that the transforming activity of the C1R/TPPAP9 cell line evolved during the transformation process and was not present in the original group of pooled papillomas.

Expression of c-myc in Mouse Skin Tumors

Primary tumor RNA from some of the same panel of mouse skin tumors plus some additional tumors not used in the transfection assay were examined for myc gene expression. Northern blot analysis of total tumor RNAs and normal and treated mouse epidermal RNAs are shown in Figure 12. There was virtually no myc expression detected in the normal or treated CD-1 mouse epidermis (pEpd 1-4) while three of the tumor derived RNAs showed a complex transcriptional pattern of myc expression (pap4, scc1 and ssc2). The endogenous mouse myc transcript is approximately 2.2-2.4 Kb. A papilloma (pap4) and two
Figure 12. Northern Blot Analysis of Total RNAs Hybridized to a 1.5 Kb Pst1 Insert of Viral myc (pmyc-2).

Total RNAs were isolated from CD-1 animals. RNAs from pooled epidermis are: lane 1, animals treated with acetone; lane 2, animals promoted with TPA; lane 3, animals initiated with ionizing radiation; lane 4, animals initiated with ionizing radiation and promoted with TPA. RNAs from papillomas: lane 1, animals promoted with TPA; lanes 2-5, animals initiated with ionizing radiation and promoted with TPA. Squamous cell carcinomas (SCC 1,2) and basal cell carcinomas (BCC 1,2,3) were all derived from animals initiated with ionizing radiation and promoted with TPA. 7s RNA was used as a control for amount of RNA loaded onto the gel. Size markers were prokaryotic and eukaryotic rRNAs.
independent squamous cell carcinomas (scc 1 and 2), all derived from CD-1 mice initiated with ionizing radiation and promoted with TPA, contained two novel transcripts. The novel transcripts in these three tumors appeared to be similar in size, approximately 3.8 and 1.8 Kb. These tumor RNAs were also probed with Ha-ras. A representative sample is shown in Figure 13. The three tumors which showed a complex transcriptional pattern of myc expression only expressed the endogenous 1.4 Kb Ha-ras transcript (lanes ppap2, scc 1 and scc 2). The Ha-ras transcript was expressed at similar levels in all tumors and epidermal samples studied. Variation in intensity of transcripts was the result of uneven loading onto the gel.

Northern dot blot analysis of additional Sencar tumor RNAs and a sample of normal and treated mouse epidermal RNAs are shown in Figure 14. There is evidence for a slight increase in myc expression in 1 out of 5 Sencar squamous cell carcinomas and in 1 out of 3 adenocarcinomas. At the present time it is not known if the tumors that show an increase in myc expression also have a complex transcription pattern as seen in Figure 12.

Amplification and Rearrangement of c-myc in Mouse Skin Tumors

Primary DNA from skin tumors, normal and treated epidermis and liver were examined for amplification and/or rearrangement of c-myc. Southern blot analysis of Bam HI digested DNAs is shown in Figure 15. The endogenous 5.6 Kb myc fragment is present in the majority of the samples examined. Evidence for rearrangements was
**Figure 13.** Northern Blot Analysis of Tumor RNAs Hybridized to a 600 bp EcoRI/BamHI Insert of Mouse Ha-ras (psp659EB₂-ras).

Total RNAs from pooled epidermis (pEpd₁); pooled papillomas (ppap₁ and pap2) squamous cell carcinoma (SCC 1,3) and basal cell carcinoma (BCC 1,2) were all derived from CD-1 animals that had been initiated with ionizing radiation and promoted with TPA. Size markers were prokaryotic and eukaryotic rRNAs. 7s RNA was used as a control for amount of RNA loaded onto the gel.
Figure 14. Northern Dot Blot Analysis of Total Tumor and Tissue RNA.

The probe was a 1.5 Kbp PstI insert of viral myc (pmyc-2). Total RNA was isolated from Sencar mice. Pooled epidermis (pEpd) are: lane 1, animals treated with acetone; lane 2, animals promoted for 60 weeks with TPA; lane 3, animals initiated with β-rays; lane 4, animals initiated with β-rays followed by TPA promotions; lane 5, animals initiated with x-rays followed by TPA promotion. Squamous cell carcinomas (SCC): lane 1 and 2 animals promoted with TPA for 60 weeks; lanes 3-5, animals initiated with x-rays followed by TPA promotion. Adenocarcinomas (Ac): lanes 1-3, animals initiated with x-rays followed by TPA promotion. 7s RNA was used as control for amount of RNA load.
Figure 15. Southern Blot Analysis of Tumor DNAs Digested to Completion with BamHI and Hybridized to a 1.5 Kb PstI insert of Viral myc (pmyc-2).

All samples came from animals that were initiated with ionizing radiation and promoted with TPA. Lanes 1-9 are from CD-1 mice and lanes 10-13 are from Sencar. DNAs are: lane 1,11, pooled epidermis; lanes 2,10, liver; lanes 3-5, papillomas; lanes 6,12-13, squamous cell carcinoma; lanes 7-9, basal cell carcinomas. Size markers in Kb were Hind III-digested λ-phage.
observed in DNAs from the CD-1 papilloma (lane 5) and squamous cell carcinoma (lane 6) that showed novel myc transcripts in Figure 12. The endogenous 5.6 Kb fragment was absent from these two samples. DNA from CD-1 epidermis treated with ionizing radiation plus TPA and corresponding liver contained the endogenous unrearranged c-myc fragment (lane 1,2). It is of interest to note that DNA isolated from Sencar epidermis initiated with β-rays and promoted with TPA showed a rearrangement similar to that observed with the two CD-1 tumors (lane 12). In this sample, a faint endogenous 5.6 Kb band was observed. Liver isolated from the same group of animals did not contain a rearranged c-myc gene (lane 10). The nature of the c-myc transcript in this Sencar epidermis is not known. It will be of great interest to examine Sencar epidermis after various treatment protocols to determine if the rearranged c-myc is just a rare occurrence or if it can be correlated with a specific treatment protocol.

Discussion
To examine the role of oncogenic activation in radiation induced mouse skin tumors and address the question of tissue specificity, tumor DNAs were examined for the presence of dominant transforming activity by the NIH3T3 focus assay (Table 10). Ninety percent of all tumor DNAs studied were positive in this assay. Dominant transforming activity was observed in all tumor types (papillomas, basal and squamous cell carcinomas and adenocarcinomas)
but not in normal or treated epidermis or corresponding liver. DNAs from NIH3T3 transfectant cell lines were analyzed using Southern blots for the presence of the three ras genes (Ha, Ki, N). If restriction sites closely linked to the transferred gene were destroyed by restriction digestion before transfection or during the transfection process, then DNA prepared from transformed foci would contain novel restriction fragments hybridizing to the gene probes of interest. Novel restriction fragments were not observed in any of the NIH3T3 transfectant DNAs examined, nor were any of the endogenous fragments amplified (Figure 8A). These data indicate that Ha-, Ki- and N-ras genes were not responsible for the observed dominant transforming activity. However, the possibility of subtle mutations in any of these three genes resulting in transforming activity without leading to genomic amplifications or novel restriction patterns could not be ruled out. To address this possibility, DNAs from primary NIH3T3 transfectants were used in Rat-2 focus assays (Table 10); DNAs from Rat-2 transfectants were then analyzed by Southern blots to look for the presence of mouse ras genes in a rat genomic background. As shown in Figure 8B the only ras genes present in the Rat-2 transfectants were the endogenous rat sequences. Thus, there is no evidence for oncogenic ras genes in any of the radiation induced mouse skin tumors examined.

It has been reported that Ki-ras is the most commonly activated ras gene in response to physical carcinogens such as
radiation (Guerrero et al, 1984b; Sawey et al, 1987). However, close examination of the data suggests that the particular program of oncogenic activation involved in tumorigenesis may vary according to cell type and/or strain of animal studied. Pellicer and colleagues (personal communication) have evidence which shows that ionizing radiation results in activation of either Ki- or N-ras in mouse thymic lymphomas. The frequency of activation of a particular gene appears to be strain dependent. Sawey et al. (1987) have recently examined a number of rat skin tumors induced by ionizing radiation and have found 3 out of 3 clear cell carcinomas and 1 out of 5 squamous cell carcinomas containing an activated Ki-ras gene, suggesting cell type specificity. However, evidence for Ki-ras activation was not observed in any of the mouse tumors induced by ionizing radiation in this study.

To further characterize the transformed phenotype of these transfectant cell lines, growth in soft agar and tumorigenicity studies were performed (Table 11). Both the percent cloning efficiency and tumorigenicity in nude mice increased in secondary transfectants over the primary transfectants from which they were derived. These data demonstrate that the transforming activity was serially passaged during subsequent rounds of transfection and further confirming the presence of transferred transforming activity. To determine if the dominant transforming activity observed in these tumors was the result of previously characterized oncogenes, transfectant DNAs were screened for the presence of
oncogenes shown to be active in the NIH3T3 assay. There was no
evidence of activation of B-lym, met, neu, raf or erbB (Figures 9-
11). As discussed earlier, the transfectant cell line C1R/TPPAP9
appeared to have acquired a novel neu restriction fragment. This
fragment was not observed in other independent primary C1R/TPPAP
transfectants or in the original tumor material, indicating that it
arose during the process of transfection. In order to characterize
the transforming activity in these tumors, restriction endonuclease
functional maps were performed. DNAs were isolated from 4
transfectant cell lines established from 4 independent squamous cell
carcinomas, digested with various restriction enzymes and then
assayed for transforming activity. The results suggest that 2 of
the 4 transfectants had a similar functional map.

The data presented here suggests that the targets for onco-
genic activation are different for chemical carcinogens and ionizing
radiation. Support for this has recently been presented by Borek et
al. (1987). Their findings show that in vitro malignant transforma-
tion of mammalian cells (hamster and mouse C3H/10T 1/2) by a single
direct exposure to x-irradiation results in the activation of onco-
genic sequences with detectable transforming activity. The oncogenic
sequences activated do not represent activated forms of the ras gene
family, which have been implicated in chemically transformed C3H/10T
1/2 cells. A better understanding of the radiation-transforming
genes will add insight to molecular mechanisms in radiation
carcinogenesis and will serve as a powerful tool to dissect steps in
the process of radiation-induced malignant transformation.

Perhaps it is not surprising that Ha-ras activation was not identified with radiation-initiated mouse skin tumors as was observed with chemically initiated mouse skin tumors (Balmain et al., 1984; Bizub et al., 1986; Quintanilla et al., 1986). In the mouse skin model of carcinogenesis, the nature and frequency of the activated oncogene appears to be dependent upon the initiating agent used but not upon the promoter (Quintanilla et al., 1986).

Proto-oncogenes are defined as genes that normally play a major role in the control of growth or differentiation and thus have the capacity when mutated or activated to become oncogenes. If one assumes that 1 percent of the genes in a mammalian genome (estimated to be approximately 50,000 genes total) are involved in growth control in some manner, then it is conceivable that the number of proto-oncogenes is larger than the current list of 40, perhaps as many as 500. If one also assumes the low LET radiation induces mutations in a random manner within the genome, then it is conceivable that three different transforming activities could be found in four independent squamous cell carcinomas. In fact, there are a number of reports in the literature of unidentified oncogenes (Fox and Watanabe, 1985; Garte et al., 1985; McMahon et al., 1986; Pulcian et al., 1987).

The damage produced by ionizing radiation results in a number of complex alterations to reactive sites in DNA. Damage occurs in many sites in the purine and pyrimidine rings destroying deoxy-
ribose residues, causing DNA double- and single-strand breaks and producing DNA protein crosslinks (Leenhouts and Chadwik, 1974; Hall, 1978; Hall and Miller, 1981). When cells are exposed to ionizing radiation, one of the most noted effects is the production of aberrant chromosomes (Wolff, 1961; Evans, 1962; Wolf and Carrano, 1986). The aberrations are the result of either simple breakage of chromosomes or the interaction of broken ends to form rearrangements. Studies over the past five years have demonstrated activation of various proto-oncogenes via specific chromosomal translocations (Dalla-Favera et al., 1982; deKlein et al., 1982; Marcu et al., 1983). The recent finding of myc activation by gene amplification and rearrangement in 9 of 12 rat skin tumors induced by ionizing radiation (Sawey et al., 1987) is of great interest. It suggests that carcinogenic agents may in certain instances directly interact with target proto-oncogenes resulting in their activation. Novel c-myc transcripts were observed in the studies presented in this chapter (Figure 12). This was seen in one out of five papillomas and two out of two squamous cell carcinomas. None were observed in the three basal cell carcinomas. All tumors were derived from CD-1 animals after an initiating dose of ionizing radiation followed by TPA promotion (Chapter 3).

Evidence for genomic myc rearrangements was observed in the DNAs from the papilloma and squamous cell carcinoma which showed novel myc transcripts (Figure 15). It is of interest to note that DNA from CD-1 epidermis treated with ionizing radiation and TPA and
corresponding liver contained only the endogenous unrearranged myc fragment. On the other hand, DNA isolated from Sencar epidermis initiated with β-rays and promoted with TPA showed a rearrangement similar to that observed with the CD-1 tumors. DNA form the liver of the same Sencar animal only contained the endogenous unrearranged myc gene. It will be important to examine Sencar epidermis after various treatment protocols to determine if the observed rearranged c-myc can be correlated with treatment regime or if it was just an isolated observation.

From the Southern blot analysis (Figure 15), it appears that the genomic myc rearrangements are very similar in the papilloma, squamous cell carcinoma and epidermis. This was somewhat surprising. It has been assumed for many years that the interaction of low LET radiation with DNA is random. There have not been any reports of potential hot spots as in prokaryotic DNA sequences treated with UV. However, the similarity of the rearrangements observed in Figure 15 and the similarity of rearrangements observed by Sawey and colleagues (1987; personal communication) in 9 out of 12 radiation-induced rat skin tumors would suggest a non-random interaction. In the mouse cellular myc gene (Figure 16), there is a 1.1 Kb region containing exon 1 and part of intron 1 where most break points occur (Bernard et al, 1983). Tumors containing break points outside the normal transcriptional unit have c-myc mRNA transcripts similar to that found in normal cells (2.2-2.4 Kb), while tumors with break points inside the transcriptional unit
Figure 16. Structure and Restriction Map of the Mouse and Human c-myc Genes.

Boxes denote exons: filled boxes, coding sequences; hatched box, untranslated exon 1; stippled box, 3'-untranslated region of exon 3. T denotes the murine region within which most translocations occur. (Bernard et al., 1983).
display altered transcripts, the size of which have been shown to vary from 2.1 to 4.3 Kb (Bernard et al., 1983). It is surmised that the altered mRNA arise from activation of cryptic promoters within the 3' half of intron 1 and/or altered splice sites. The data presented in Figure 12 and 15 support this deduction. If the break points occur at the Bam HI site located in intron 1, alterations in c-myc transcripts would be expected. This can be proven by probing the c-myc mRNA with an exon 1 specific probe. Unlike normal c-myc mRNA, transcripts resulting from break points in intron 1 would not be labeled by an exon 1 probe. This still does not explain the similarities in genomic rearrangements and mRNAs from the three different tissues. It is possible that sequences in the first intron of c-myc are very sensitive to the effects of ionizing radiation. Clearly there is a need to further characterize the molecular alterations involved in the myc rearrangements and to examine more tumors of both Sencar and CD-1 origin and study the effect of ionizing radiation on myc activation in the epidermis.

To summarize, the data presented in this chapter show that radiation-induced mouse tumors result in the activation of oncogenic sequences with detectable transforming activity in two recipient cell lines, mouse NIH3T3 and Rat-2 cells. The oncogenic sequences activated in these tumors are unique. They do not represent activated forms of the ras gene family, which have been implicated in chemically initiated mouse skin tumors (Balmain et al., 1984; Bizub et al., 1986; Quintanilla et al., 1986). The oncogenic
sequences also do not represent activated forms of erbB, B-lym, met, neu or the raf oncogenes, which have been shown to be active in the transfection assay. The work presented here also provides evidence for novel c-myc transcripts and corresponding genomic rearrangements in a few of the tumors studied.

Molecular cloning and sequence analysis of the radiation-induced transforming genes and the rearranged cellular myc genes should add to our growing knowledge of the molecular mechanisms of radiation carcinogenesis.
CHAPTER 6

CONCLUSIONS

A large body of evidence indicates that carcinogenesis is a process involving multiple independent steps. Epidemiological studies have suggested that cancer arises in proportion to a multiple power of elapsed life time. Pathological studies show that tumors progressively acquire new phenotypes by passing through a series of distinct stages such as anaplasia, metaplasia and finally neoplasia. Moreover, in many systems, experimental induction of a tumor requires at least two distinct types of stimulus, such as an initiator and a promoter. At the cellular level, evidence is emerging which suggests that each step may require activation of distinct genes and the final phenotype may require concomitant expression of many previously activated genes. According to this model, genetic damage changes a cell's responsiveness to growth and/or differentiation signals, resulting in tumor formation. Additional alterations are thought to be required for progression of the tumor to a malignant phenotype.

The multi-step model of carcinogenesis accommodates many aspects of radiation-induced tumorigenesis, specifically the long latent period and age dependency. Evidence of a stepwise evolution of radiation-induced neoplasms comes from the ability of hormones
and other promoting agents to enhance the induction of such growth when administered after irradiation. The incidence of breast tumors increased greatly when x-rays were followed by mammotrophic (Furth, 1975) or estrogenic (Sheallabarger, 1981) stimulation. Endocrine stimulation also promotes the pathogenesis of radiation induced murine harderian gland tumors (Upton, 1984). Other examples of tumor promotion include the enhanced induction of thymic lymphomas and hepatocellular carcinomas in mice and rat kidney tumors by administration of urethane, carbon tetrachloride and contralateral nephrectomy after exposure to ionizing radiation (Upton, 1984).

Data presented in this dissertation shows that ionizing radiation can act as an initiator in mouse skin. The tumor promoter, TPA, greatly enhances the formation of squamous cell carcinomas when given after a single subcarcinogenic dose of ionizing radiation in both Sencar (Chapter 2) and CD-1 (Chapter 3) animals. For a given dose of ionizing radiation and TPA promotion, there was no difference in squamous cell carcinoma induction between these two strains. However, TPA pretreatment was much more effective in decreasing the latency period and increasing the tumor incidence in Sencar animals. Sencar mice are 2-to-3 fold more sensitive to TPA promotion than CD-1 animals (DiGiovanni, Slaga and Boutwell, 1980). It is attractive to speculate that the increased sensitivity to tumor induction in Sencar animals is the result of the skin's response to TPA. A strain difference in radiogenic-induction of non-squamous tumors was also observed. Fibrosarcomas
were the most common non-squamous tumor in Sencar animals, while basal cell carcinomas occurred at a higher frequency in CD-1 mice.

The only other experimental animal system in which basal cell carcinomas occur are in rat skin exposed to carcinogenic doses of ionizing radiation. The incidence of basal cell carcinomas was dependent on the dose of ionizing radiation and independent of TPA promotion. This was in contrast to squamous cell carcinomas whose incidence was dependent on the interaction of ionizing radiation and TPA. The mechanism of induction of these two malignant tumor types and how ionizing radiation interacts with the skin to induce these lesions remains unknown. The data indicate that the interaction between ionizing radiation and TPA in vivo (when analyzed in terms of tumor response) is complex. The final outcome is dependent upon the strain of animals, total dose of ionizing radiation delivered, length of promotion and target cells involved in the tumor response.

The findings discussed in Chapters 2 and 3 revealed an unexpectedly high percentage of papillomas progressing on to squamous cell carcinomas. This led to speculation that, in addition to its weak initiating activity, ionizing radiation may be active in malignant progression. In Chapter 4, a three-stage (initiation, promotion, progression) experiment was discussed. Papillomas were induced in CD-1 animals with a single subcarcinogenic dose of MNNG followed by TPA promotion. Ionizing radiation (8 fractions of 1 Mev electrons) was used in the third stage. Conversion of
papillomas to squamous cell carcinomas was three times greater in animals treated with ionizing radiation than in papilloma-bearing animals treated with TPA. In this experiment and another similar experiment (in Sencar animals) which is ongoing and not discussed in this dissertation, the promoting effects of ionizing radiation have been examined. Under the fractionated conditions examined in Chapter 4, no new papillomas developed over the course of the study. In fact, papillomas treated with ionizing radiation were observably smaller than papillomas present on tumor bearing animals not treated with ionizing radiation. The mechanism by which ionizing radiation enhances malignant progression is currently unknown.

The correlation between mutagenicity and carcinogenicity of certain organic chemicals has refocused attention on models of carcinogenicity that relate the initiation of neoplastic growth to mutational events. Induction of cancer by ionizing radiation assumes that radiation acts by inducing somatic mutations which result in alteration of cellular growth and differentiation controls. It is attractive to postulate that radiation-induced chromosomal aberrations can cause cancer. Most breaks produced by ionizing radiation restitute, or become repaired. A smaller proportion remain unrepaired and can lead to the formation of fragmented chromosomes. Some of the broken chromosomes undergo a form of misrepair in which broken ends from different breakpoints rejoin with one another to form two-break alterations. These include: translocations, inversions and interstitial deletions.
These aberrations require the interaction of two independent breaks each of which is produced in direct proportion to the dose (Wolf and Carrano, 1986). The breaks that interact to form these aberrations must be induced very close to one another within the cell nucleus. Although it has been shown that single breaks are produced randomly within the nucleus, aberrations caused by the interaction of two-breaks are thought to be produced non-randomly (Wolf and Carrano, 1986).

The types of radiation-induced genomic alterations that might induce a malignant transformation must be consistent with cellular viability. Unstable alterations such as dicentrics, rings and large deletions that result in cellular lethality would not contribute directly to a developing tumor. Stable alterations such as balanced translocations, inversions, small deletions and point mutations could result in alterations in growth and differentiation regulatory genes resulting in cellular transformation. This could occur by activation of proto-oncogenes and/or inactivation of tumor suppressor genes. Suppressor gene products may interact with, modulate, or compete with the oncogene products or they may at different points within a chain of events leading to tumorigenicity. Genetic damage to suppressor genes can alter the normal function of their gene products resulting in abnormal growth and/or differentiation (Stanbridge, 1984; Sager, 1986).

Molecular studies have now demonstrated that various cellular homologs of retroviral oncogenes are located next to break points of
specific translocations, and may be activated by these alterations (Dalla-Favera et al., 1982; deKlein et al., 1982; Harris et al., 1982; Marcu et al., 1982; Shen-Ong et al., 1982; Taub et al., 1982). These activated genes have been identified as genetic targets which play a key role in carcinogenesis (Varmus, 1984). The development of DNA-mediated gene transfer techniques has made it possible to detect specific genes capable of inducing phenotypic changes in recipient cells (Wigler et al., 1978). Most of the transforming genes detected in the NIH3T3 assay have been found to be mutated members of the ras gene family (reviewed: Bishop, 1985). The actual function of these transforming genes in tumor cells is unknown. They are postulated to be involved in induction and maintenance of the malignant phenotype. Results obtained from mouse skin and mammary gland tumors induced by chemical carcinogens suggest that activation of one oncogene (Ha-ras) is not sufficient to cause malignancy in these organs (Zarbl et al., 1985; Bizub et al., 1986; Quintanilla et al., 1986). Only after cell proliferation induced exogenously by promoters (abnormal) or by hormonal stimulation (normal) do some of these cells assume anaplastic characteristics. In the mouse skin model the activated Ha-ras gene is found both in the benign papilloma and squamous cell carcinoma. In some squamous cell carcinomas there is evidence that the activated allele of Ha-ras is amplified and the normal allele is absent (Quintanilla et al., 1986). These results are consistent with malignant progression being a consequence of more than one lesion
and suggest a role for ras in early stages of tumor progression.

Studies of ionizing radiation-induced mouse thymomas (Guerrero et al., 1984b) and rat skin tumors (Sawey et al., 1987) have reported the activation of c-Ki-ras in some neoplastic cells. Studies of in vitro malignant transformation of hamster and mouse cell lines by exposure to x-rays results in activation of previously undescribed oncogenic sequences (Borek et al., 1987). These sequences do not represent activated forms of the ras family or any other oncogenes which have been shown to be active in the NIH3T3 transfection assay. The findings of Borek and colleagues (1987) are similar to those presented in Chapter 5. It seems likely that different cell types use different pathways to control cell growth and within a particular cell type there are multiple pathways which direct cellular growth. Weinstein (1987) predicts that the genomes of higher organisms contain several hundred proto-oncogenes. Vertebrates contain at least 30 cell types, and it is likely that the growth of each cell type is under control of several growth factors (Giulian et al., 1986; Gousten et al., 1986; LaBeau et al., 1986). Each growth factor in turn occupies a specific cell receptor, which might also be considered to be a proto-oncogene. If one assumes additional genes are required to mediate signal transduction for each receptor and that the nucleus contains specific transcriptional factors that control the expression of responsive genes, the number of proto-oncogenes is rather large. Despite the large repertoire of proto-oncogenes, it appears that
certain members become activated preferentially or are strongly selected for during tumorigenesis. It is probable that the current assay techniques and recipient cell lines used in the DNA-mediated gene transfer experiments are selective for specific gene families. As new assays are developed and new recipient cell lines utilized, one would predict a vast increase in the rate of identification of cellular oncogenes.

It was of interest to find altered c-myc transcripts and corresponding genomic rearrangements in several radiation-induced mouse skin tumors (Chapter 5). Sawey et al. (1987) have recently observed amplification and/or genomic rearrangements in 9 out of 12 radiation induced rat skin tumors. Alteration of cellular myc is most commonly implicated in the genesis of certain lymphoid tumors of mouse and man. However, it has been implicated in a variety of other tumors (reviewed in: Klein and Klein, 1985; Cory, 1986). The fact that similar rearrangements were observed in mouse skin tumors of different histologic-phenotype and treated (radiation plus TPA) Sencar epidermis (Chapter 5) suggests potential sequence hot spots for radiogenic-induced DNA damage. Molecular cloning and characterization of myc genes from these tissues will contribute to the further understanding of mechanisms involved in induction of radiogenic DNA damage and subsequent carcinogenesis.

In spite of enormous progress accomplished during the last five years in molecular carcinogenesis, an understanding of the molecular intricacies of neoplastic development is far from
complete. Carcinogenesis most likely results from a combination of genetic and epigenetic alterations affecting the capacity of cells to overcome their normal proliferative and differentiation restraints and escape immunosurveillance. The use of animal tumor models has led to important insights into molecular mechanisms by which carcinogenic agents may activate cellular oncogenes. The mouse skin model using ionizing radiation as an initiator is an excellent model for the study of activation of oncogenes in individual etiologically defined tumors. This model will serve as a powerful tool to dissect steps in the process of radiation-induced malignant transformation.

In summary the major new findings of this dissertation are:
1. Ionizing radiation is a weak initiator in mouse skin; 2. The incidence of squamous cell carcinomas is dependent on the initiating dose of ionizing radiation and duration of TPA promotion; 3. The incidence of basal cell carcinomas is dependent only on the dose of irradiation; 4. Ionizing radiation can alter the malignant conversion rate when delivered to papilloma bearing mice; 5. The majority of radiation induced skin tumors have dominant transforming activity as measured by the NIH3T3 and Rat-2 focus assay; 6. Southern blot hybridization to ras (Ha, Ki, and N), β-lym, erbB, met, neu, and raf indicate that these genes are not involved in the observed transforming activity; 7. The oncogenic sequences activated in these tumors appears to be unique; 8. Evidence for novel c-myc transcripts and corresponding genomic rearrangements in a few of the tumors studied was observed.
REFERENCES


Ames, B.N. (1979) Mutagen will be used in its broad sense to induce clastogens and other DNA damaging agents. *Science* 204:587-592.


chromosome translocation in B-lymphoid tumors. EMBO J. 2:2375-2383.


Nuss, R., van Ooyan, A., Rijsewijk, F., van Lohuizen, M.,


