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SEQUENCING PCR AMPLIFIED *RAS* GENES TO DETERMINE THE IMPORTANCE OF MUTATIONAL ACTIVATION OF ENDOGENOUS *RAS* GENES TO THE MALIGNANT TRANSFORMATION OF HUMAN FIBROBLASTS

By

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ABSTRACT

SEQUENCING PCR AMPLIFIED *RAS* GENES TO DETERMINE THE IMPORTANCE OF MUTATIONAL ACTIVATION OF ENDOGENOUS *RAS* GENES TO THE MALIGNANT TRANSFORMATION OF HUMAN FIBROBLASTS

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Activation of *ras* proto-oncogenes by mutation at specific codons has been documented in a variety of human malignancies. To determine the extent to which *ras* activation plays a role in the malignant transformation of human fibroblasts, I sequenced the coding region of the H-, K-, and N-*ras* genes from a series of human fibroblast cell lines, including normal diploid human fibroblasts, two morphologically normal non-tumorigenic infinite life span cell strains originated in this laboratory, spontaneous and carcinogen-induced malignant transformants of the latter, and cell lines derived from human fibrosarcomas. Direct sequencing of the PCR product was carried out using the dideoxy chain termination method. A total of twelve cell lines or strains were analyzed for *ras* mutations. Of the eight that are malignant two fibrosarcoma-derived cell lines contained an activating *ras* mutation, a frequency of 25%. One mutant cell line, 8387, has a G to C transversion that causes an Ala to Pro amino acid substitution at codon 146 in one allele of the K-*ras* gene. This same mutation has been reported to activate K-*ras* in Hut-14 cells, an 8387 derivative. The other mutant cell line, HT1080, has a C to A transversion that causes a Gln to Lys amino acid substitution at codon 61 in one allele of the N-*ras* gene that

has been reported previously. The remaining cell lines or strains contain no mutations in *ras*. The mutation frequency in the fibroblastic cells tested in this study is consistent with one other report on *ras* activation in human sarcomas in the literature. My results show that although *ras* oncogenes can play a role in the development of fibrosarcomas in humans, some other changes must be able to substitute for *ras* activation in the malignant transformation of human fibroblasts. Also, methods of detecting *ras* activation that concentrate on the "classic" activating codons may miss activated genes. Further studies are planned to test for alterations elsewhere in the *ras* p21 signalling pathway using cells that contain only wild-type *ras* genes.

This work is dedicated to my parents, Howard and Margaret Scheid, to my first role model, Mrs. Patricia Pregitzer, and to my partner, Cindy, for their caring and support.

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INTRODUCTION

Malignant transformation of cells has been established through a variety of epidemiological and experimental studies to be a multi-step process. Through the accumulation of stable heritable changes an individual cell loses regulation of growth and differentiation, and gains the ability to invade into adjacent, normal tissue and to metastasize to distant body sites. The overall goal of investigators who study this process is to understand the nature of the events that result in transformation, and to find the means either to interrupt the process before a patient becomes ill with a malignancy, or to treat a malignant tumor once it has developed.

In the course of decades of investigation, researchers have identified many genes coding for proteins that are involved in regulation of growth and differentiation in normal cells. These genes have been shown to be altered in characteristic ways in tumor cells, and therefore to encode proteins that no longer provide normal regulatory function. These genes have been categorized into one of two groups: dominant-acting oncogenes and tumor suppressor genes. The dominant-acting oncogenes need only one of two alleles to be activated in order to have transforming activity. Within this group are genes encoding proteins that are associated with the plasma membrane (e.g., growth factors and growth factor receptors), genes that encode cytosolic proteins (e.g., protein kinases), and genes that encode nuclear proteins (e.g., DNA binding

proteins)(Bishop, 1983). The tumor suppressor genes exert their transforming effect only when both alleles are altered such that no functional protein is produced. The majority of the products of tumor suppressor genes are found in the nucleus, bind to DNA, and act as regulators of gene expression (Levine and Momand, 1991).

The various *ras* genes are members of the membrane-associated dominant-acting oncogenes. They encode proteins classified as "G-proteins" because their activity is regulated by changes in tertiary structure brought about by binding to the guanine nucleotides GTP and GDP. The proteins encoded by these genes have a molecular weight of 21kDa, are anchored to the internal surface of the plasma membrane, and function as transducers of the mitogenic signals from protein growth factors (Barbacid, 1987). Ever since the discovery that the transforming gene in the acutely transforming Harvey murine sarcoma virus had a normal cellular homolog (Parada et al., 1982), these genes, and their products, have been the subject of intense study. It is now known that activation of the *ras* genes by mutation at specific codons is a very common event in some types of human malignancy and a relatively rare event in others (Bos, 1989). Whether the source of this variability reflects differences between cell types in the process of malignant transformation, is a function of limited sensitivity of available methods, or is the result of some combination of the two, is unclear.

This study was undertaken to determine the extent to which *ras* activation occurs in the malignant transformation of one type of cell, i.e., human fibroblasts. Human and rodent fibroblasts in culture are commonly used as model systems to study the process of malignant transformation, and are readily transformed by insertion of activated *ras*

genes. Therefore, it is important to determine whether activation of endogenous *ras* genes is commonly involved in the transformation of such cells. With the advent of polymerase chain reaction (PCR), it became possible to amplify specifically the entire coding region of the *ras* genes, and use this amplified fragment as a template for direct sequencing. Previous efforts to determine the frequency of *ras* activation have concentrated on the sequence of the specific codons that are known to cause transformation to focus formation in mouse NIH-3T3 cells. However, it is possible that activating mutations that do not cause transformation in that focus assay exist. Therefore, sequencing the entire coding region provides the most sensitive means to determine whether or not *ras* genes have been activated.

This first chapter of this dissertation reviews the literature on the process and mechanisms involved in malignant transformation, and specifically the role of *ras* genes in that process. The second chapter is a paper that will be submitted to the journal Molecular Carcinogenesis reporting the research I carried out for this dissertation and is written in the format required for that journal. Finally, the Appendix section gives a review of recent data on the signal transduction pathway involving ras p21, and places the results of this thesis project in the context of these new data.

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CHAPTER 1

LITERATURE REVIEW

I. Theory of Multi-Step Carcinogenesis

Cancer cells have been reported to differ from normal cells by many various characteristics. Among the most fundamental of these are the loss of normal growth control and the acquisition of infinite life span leading to the ability of the malignant cells to invade into normal tissue and/or metastasize to distant body sites. The process by which normal cells become malignant is considered to consist of several steps. Through successive stable heritable changes and clonal expansions, a cell gains the ability to divide without requiring exogenous growth factors, to continue to divide without reaching senescence, and to express protein products such as proteolytic enzymes that allow invasion and metastasis. These and other changes collectively contribute to malignancy. This theory of multi-step carcinogenesis is supported by several kinds of studies, including epidemiological, experimental, and clinical.

A. In Vivo Evidence in Support of a Multi-Step Theory of Carcinogenesis

1. Epidemiological

Peto (1977) studied epidemiological data on the incidence of several kinds of cancer. He was particularly interested the role of cigarette smoking in lung carcinoma. The data showed that a linear dose-response relationship exists between daily cigarette consumption and lung cancer incidence, and the extra incidence of lung cancer after an individual quits smoking stays approximately constant instead of continuing to rise. By studying these data in the context of several mathematical models, he found that the best fit was the multi-step model. He concluded that multi-step models would provide the most useful insights into the origins of cancer.

In 1981 Moolgavkar and Knudson developed a two-stage model of carcinogenesis. The first stage is the result of an event leading to uncontrolled cell proliferation, and the second stage is a result of an event that occurs within one of the proliferating cells and gives rise to a malignant cell that grows into a clinically apparent tumor. They showed that this model was consistent with epidemiological data on age-specific cancer rates for children and adults, on smoking and lung cancer, and on heredity and exposure to hormones and radiation in breast cancer.

In 1986 Kaldor et al. obtained and analyzed data on lung and nasal sinus cancer death rates in a group of nickel refinery workers. They concluded that calculations of excess mortality and risk are consistent with a multi-step model. In this model, type of hazard exposure, time of initial exposure, and duration of that exposure are variables that can be

used in calculating risk. Clearly, epidemiological methods have contributed to formulating multi-step models of malignant transformation.

2. Experimental

Another source of support for multi-step models of malignant transformation comes from the induction of cancer in experimental animals. A typical example is the 1964 study of Boutwell, who induced squamous cell carcinomas in the skin of susceptible mouse strains by treating once with a sub-carcinogenic dose of dimethylbenz(a)anthracene (DMBA), followed by repeated applications of the-non-carcinogenic agent, 12-O-tetradecanoylphorbol-13-acetate (TPA). Neither agent alone was sufficient to induce malignant transformation. Transformation followed a progressive course in which benign papillomas appeared initially, and foci of carcinoma developed from within the papillomas. In this experimental system, DMBA is called the initiator, since it induces a permanent change within a cell, and TPA is called a promoter, since it is required for the effect of this sub-carcinogenic dose to become apparent.

To determine the mechanisms by which chemicals induce malignant transformation of mouse skin, Verma et al. (1982) induced tumors in mice by two methods. The first was the two-agent initiation/promotion regimen of Boutwell. The second was the single-agent regimen consisting of either a single treatment with a large dose of DMBA, or multiple treatments with an intermediate dose of DMBA (as compared to the dose used in the two-agent regimen). They compared the pattern of tumor formation induced by the two regimens, and found that the two-agent protocol results in the typical pattern of rapid papilloma induction and delayed appearance of foci of carcinoma. In contrast, the single-agent protocol results in

fewer papillomas appearing after long latent periods, but more rapid and frequent appearance of carcinomas. They also found that retinoic acid specifically inhibits tumor formation by the two-agent protocol but not by the single-agent protocol. 7,8-benzoflavone, an inhibitor of the metabolic activation of DMBA, inhibited tumor formation in the single-agent protocol; however, the effect of 7,8-benzoflavone on tumor induction by the two-agent protocol was not determined. Since the patterns of tumor formation and inhibition for the two regimens differ, these investigators concluded that the mechanisms by which DMBA and TPA promote tumor formation are different. In addition, they speculated that promotion by DMBA is due to a regenerative hyperplasia following the tissue damage that is known to be caused by this agent.

3. Clinical

The induction of tumors in humans is clearly unethical. However, rare situations in which the susceptibility to a specific type of tumor is inherited offers the possibility of studying carcinogenesis in humans. The disease familial adenomatous polyposis (FAP) has a number of characteristics that make it ideal for such studies. This is an autosomal-dominant genetic disease in which patients develop hundreds of adenomatous polyps in the colon and rectum. One or more of these polyps inevitably progresses to invasive colorectal carcinomas. Vogelstein et al. (1988) studied events associated with the progression of these tumors in two groups of patients, one with FAP and the other with sporadic colon carcinoma. They assayed the cells from individual polyps for four specific genetic changes that have a documented association with colorectal neoplasia, namely, the presence of mutationally activated *ras*

genes, and allelic deletions of chromosomes 5,17, and 18. They found that polyps displaying a more-transformed phenotype contained cells with more alterations than did cells from less-transformed polyps. The least-transformed adenomas typically had only undergone one change, most often *ras* activation, whereas carcinomas exhibited *ras* activation as well as deletions in all three of the chromosomes cited above. In the more-transformed adenomas an intermediate number of changes could be detected. From their data they concluded that 1) a number of changes are required for malignant transformation of colonic epithelial cells, 2) the specific changes they observed do not substitute for each other since in most transformed groups of cells, all four of the changes can often be found, and 3) some carcinogenic change(s) must exist that can substitute for the four they studied, since all four alterations were not necessarily present in a carcinoma, and other changes have been reported.

B. In Vitro Evidence in Support of a Multi-Step Theory of Carcinogenesis

1. Early Experiments

Early in vitro transformation studies indicated that carcinogenesis was not the complex process predicted by in vivo results. As early as 1941, Gey reported the occurrence of spontaneous malignant transformation in cultured rat fibroblasts. Earle (1943) reported similar results with mouse fibroblasts in culture. However, by the late 1960's it was recognized that certain conditions of the existing in vitro culture methods (serum, light, oxygen levels) can induce transformation. Parshad et al. (1978) reported an experiment in which cultured mouse fibroblasts

were grown either in the presence or in the absence of fluorescent light. The cells grown in fluorescent light exhibited a significantly increased incidence of double strand chromosome breaks, and this damage could be decreased by the addition of reducing agents such as catalase and glutathione to the medium. The authors proposed that hydrogen peroxide produced in the medium exposed to light is responsible for the genetic damage. This could explain the frequency of so-called spontaneous transformation found by other researchers. To test this hypothesis, Sanford et al. (1979) studied the effects of fluorescent light and oxygen levels on the amount of DNA damage and the frequency of malignant transformation in cultured mouse fibroblasts. They showed that fibroblasts exposed to fluorescent light and atmospheric levels of oxygen exhibit more chromosome damage and an increased frequency of malignant transformation compared to fibroblasts that are shielded from light and grown at lower oxygen concentration.

It also appeared from early experiments that the malignant transformation of cells by infection with transforming viruses was a single step process. Halberstaedter et al. (1941) demonstrated that infection with a single agent, the *Rous* sarcoma virus, was sufficient to cause morphological transformation of chick fibroblasts in culture. Similar results were reported for infection of rat fibroblasts with the Harvey sarcoma virus. However, it is now recognized that what appeared to be a single event, i.e. viral infection, is in reality at least two events. The transforming viruses carry activated forms of cellular proto-oncogenes, for example, H-*ras* in the Harvey sarcoma virus (Parada et al., 1982). Also, the construction of these viruses places the genes under the transcriptional control of the viral long terminal repeats (LTR),

typically causing enhanced expression of the gene(s) carried by the virus compared to the expression levels that would be observed for the corresponding endogenous gene controlled by its endogenous promoter.

2. Current Experiments

A series of in vitro experimental systems is currently being used to gain a clearer understanding of the events involved in malignant transformation. These include Syrian hamster embryo (SHE) cells, human epithelial cells, and human fibroblasts.

a.) Experiments using Syrian hamster embryo cells

In 1978 Barrett and Ts'o provided support for the multi-step theory of carcinogenesis in their work with cultured Syrian hamster embryo cells exposed to the chemical carcinogen, benzo(a)pyrene. Cells in bulk culture were exposed to benzo(a)pyrene, then maintained in bulk culture conditions for up to seventy-five population doublings. Periodically during this time, cells from the bulk culture were cloned and then assayed for morphological transformation, fibrinolytic activity, and ability to grow in soft agar. Within two weeks of exposure to this agent, some cells became morphologically transformed (abnormal appearance and organization, increased cell density) and showed enhanced fibrinolytic activity. However, only after 32-75 population doublings post-exposure were some cells in the population found that displayed the ability to grow in soft agar (anchorage independence). Each of these populations that exhibited anchorage independence proved capable of forming tumors in test animals after a long latent period. The requirement for an extended period of growth after treatment in order to obtain malignant cells indicates that

subsequent events are needed to fully transform these cells.

b.) Experiments using human epithelial cells

Fewer examples of in vitro transformation exist for human epithelial cells systems owing to the historic difficulty in generating epithelial cell cultures. One problem was that serum-containing media promotes the overgrowth of fibroblasts when primary cultures are being cultivated. With the advent of serum-free medium, the establishment of epithelial cells became less complicated. Using such a medium, Rhim et al. (1985) found that successive infection with two different transforming viruses is required in order to fully transform cultured primary epidermal keratinocytes. When these cells were infected with a hybrid of adenovirus 12 and simian virus 40 (SV40), they acquired an infinite life span and exhibited abnormal morphology, but they were not able to form tumors after injection into athymic mice. However, when these partially transformed cells were infected with the Kirsten murine sarcoma virus, they formed foci (localized areas of piled up cells), grew to a higher saturation density in culture, showed anchorage independence, and formed progressively growing tumors within three weeks of injection into athymic mice.

Another example of multi-step transformation of human epithelial cells is a study by Stampfer and Bartley (1985), who exposed primary cultures of normal human mammary epithelial cells to the carcinogen benzo(a)pyrene. Following exposure, some cells displayed an extended life span and morphological changes. Two immortal cell lines arose from these extended life span cells. These two lines were dissimilar in morphology, karyotype and surface protein expression, but both were shown to be

progeny of the normal parent line. Although the immortal cells displayed some of the characteristics of malignant mammary cell lines, they formed colonies in soft agar only at a very low frequency and were not tumorigenic. In later work with these partially transformed cell lines, Stampfer and colleagues (Clark et al., 1988) reported that the presence of both the SV40 T antigen and *ras* oncogenes (inserted by retroviral infection) was required to complete the malignant transformation of the immortal cell lines.

c.) Experiments using human fibroblasts

One example of transformation using cultured human fibroblasts is a study by Namba et al. (1986), who induced malignant transformation of human fibroblasts by repeated exposure to ^{60}Co gamma radiation, followed by infection with the Harvey murine sarcoma virus. Repeated treatment with ^{60}Co resulted in immortal cells that had multiple karyotypic abnormalities, but were neither anchorage independent nor tumorigenic. However, when the immortal fibroblasts were infected with the Harvey murine sarcoma virus, some became anchorage independent and tumorigenic in athymic mice.

Studies using normal human fibroblasts in culture were also carried out in our laboratory. Normal diploid human fibroblasts with a finite life span were transfected with a plasmid carrying a selectable marker and an activated H-*ras* oncogene (Hurlin et al., 1987). The plasmid was engineered to allow over-expression of the oncogene. Transfected cells that expressed the oncogene were morphologically transformed, focus-forming, and anchorage independent. The transformed cell lines were expanded in culture for tumorigenicity studies. However, during this

expansion the cells showed decreased ras p21 expression and reversion to normal morphology. These revertants did not form foci or colonies in soft agar, nor were they tumorigenic. All the transformants senesced after 40-50 population doublings as is typical for normal fibroblasts. Once an immortal fibroblast line had been generated (Morgan et al., 1991), transfection of this activated *ras* oncogene (Hurlin et al., 1989) or other *ras* oncogenes (Wilson et al., 1990; Fry et al., 1990) malignantly transformed these cells.

The above studies provide strong evidence in support of the concept of multi-stepped carcinogenesis. In order to understand malignant transformation more completely, one must consider the changes that occur during the process. The next section of this chapter will outline the genetic mechanisms that play a role in malignant transformation.

II. Genetic Basis for Malignant Transformation

A. DNA Tumor Viruses

Infection of permissive mammalian cells by members of the DNA tumor virus family typically results in lysis of the affected cell. Control of the lytic cycle is mediated by the proteins coded by viral genes that are transcribed early after infection (early region genes). They act to disrupt the normal pattern of protein expression in the host cell and to regulate the production of viral progeny. When infection of non-permissive cells occurs, the host cells are not lysed, but on rare occasions may become transformed. Transformation is thought to be such a rare event because in order for the virus to permanently disrupt normal growth of the host, the early genes must continually express proteins.

This requires at minimum the integration of that 5' regulatory region of the virus into the host genome such that the early protein(s) are expressed. The study of these viruses (adenovirus, papovavirus, and herpes virus) at the genetic and molecular level has revealed the identity of many of these transforming proteins, and has yielded an understanding of the mechanisms by which their interactions with the host proteins release the host cell from its normal growth regulation.

1. Adenovirus

The adenovirus (Ad) family is comprised of multiple serotypes. None have been linked to cancer in humans, but each has the capacity to transform human and animal cells in culture and to induce tumors in animals. Three serotypes, Ad 2, 5, and 12, are similar in the sequence and organization of their early region genes. Several studies illustrate the role of the Ad early genes in transformation. In a series of experiments in which fragments of adenovirus genome were transfected into rodent cells in culture (reviewed by Petterson and Roberts, 1986), eight percent of the 5' region, known as E1, was determined to contain the necessary and sufficient transforming sequences. E1 has been divided into two genes, *E1A* and *E1B*, each contributing uniquely to the process of malignant transformation.

E1A encodes two major proteins, 243 and 249 amino acids respectively. These proteins are found in the nucleus of the cell, share amino and carboxyl terminal sequences, but differ in their central region by 43 amino acid residues that are unique to the larger protein. Domains of high sequence similarity among several serotypes have been identified and associated with specific transforming properties. Domains 1 and 2,

contained in both protein products, act to immortalize the host cell (perhaps in part as a result of binding to the product of the retinoblastoma susceptibility gene, *RB* (Whyte et al., 1988)), induce production of growth factors (also implicated by Quinlan et al., 1988 in immortalization), and cooperate with *ras* oncogenes to transform primary cultured cells to malignancy (Ruley, 1983). Domain 3 (unique to the 249 amino acid protein) is known to act as a transcriptional trans-activator for both viral and cellular genes.

The *E1B* gene encodes two major polypeptides. The first, 179 amino acids in length (19kDa), is thought to act both as a growth factor and as a regulator of virion production. In studies of viruses carrying mutations in the region encoding this protein, Subramian et al. (1984) described a deletion mutant, *cyt*, that caused extensive destruction of human KB cells upon infection. This mutant was also significantly less-capable of inducing cellular transformation in primary rat embryo fibroblasts and in the established rat fibroblast cell line CREF, suggesting that this locus is important in regulating the balance between these two outcomes of infection. Further characterization of these mutants (Subramian et al., 1985) revealed that in the rare instances when the *cyt* mutant did transform cells, the transformants could not grow as well in low calcium medium as those transformed by wild-type virus. This suggested that the wild-type protein acts as a growth factor and decreased the need for the 1.0mM calcium typically utilized by those cells. Calcium is known to act as growth factor in normal fibroblasts (Schilz, 1988), whereas the ability of cells to grow in low calcium is shared by many transformed cells. The other major E1B protein, 495 amino acids in length (55kDa), is phosphorylated on serine and threonine, and has been shown to

bind the *p53* gene product (Sarnow et al., 1982). The *E1B* gene alone is incapable of transforming cells, but can act to augment and cooperate with the products of *E1A*.

2. Papova Viruses

The papova virus family is divided into two subfamily groupings, the polyoma viruses, including polyoma and SV40, and the papilloma viruses. Although many other viruses are included in the polyoma subfamily, polyoma and SV40 have been studied extensively with regard to transformation and will be used for illustrative purposes here.

The genomic structure and transforming mechanisms of polyoma and SV40 are very similar. The transforming region of both viruses has been mapped to the early region. By alternative splicing, the early gene gives rise to three mRNA species in polyoma (large T, middle T, and small t) and two mRNA species in SV40 (large T and small t). The products of these messages share amino terminal domains but have separate carboxyl terminal domains. In the course of lytic infection, these proteins mediate the expression of viral and host genes for the purpose of producing progeny virus. During transformation, they act to deregulate the host cell in two important aspects, immortalization and release from dependence on exogenous growth factors.

a.) Polyoma virus

i. Large T antigen

Wild-type large T antigen of polyoma virus was shown by Rassoulizadegan et al., (1983) to be sufficient to establish continuous cultures from primary rodent fibroblasts. Also, cells that had been transfected with a plasmid expressing a temperature-sensitive mutant of large T antigen went into growth arrest at non-permissive temperatures, showing that expression of a functional large T was necessary for immortalization. Furthermore, using a sequential transfection protocol, fibroblast cell lines that were established by transfection with a vector containing the *large T* gene could be transformed further to focus formation by expression of middle T protein. Previous studies had shown only a transient transformation of primary fibroblast cultures by middle T antigen alone. Harlow and colleagues (Larose et al., 1991) recently showed that large T antigen binds the retinoblastoma susceptibility gene product (RB), and that mutants defective in RB protein binding also lack the capacity to immortalize primary rat embryo fibroblasts. This provides a plausible explanation for the mechanism by which large T protein immortalizes primary cells. However, Larose et al. (1991) did find some specific mutants that retained some RB protein binding capacity but were still unable to immortalize primary rat embryo fibroblast cultures, suggesting that some other property of large T is also required to immortalize primary cells.

ii. Middle T antigen

Middle T appears to release an established cell from normal growth control. The phosphatidyl inositol/protein kinase C signal transduction pathway involving $pp60^{c\text{-}src}$ is normally regulated by phosphorylation of carboxyl terminal residues, presumably as part of the response pathway to exogenous growth factor stimulus (Gould et al., 1988). Cheng et al. (1988) created several deletion and substitution mutants of $pp60^{c\text{-}src}$ and found that phosphorylation of tyrosine 527 is critical for down-regulation of kinase activity. By co-immunoprecipitation they showed that middle T binds $pp60^{c\text{-}src}$ and prevents phosphorylation of this particular residue. This change in phosphorylation pattern correlates with increased transforming potential, as measured by the induction of focus formation. This suggests that middle T stimulates growth by releasing the cell from its usual dependence on exogenous protein growth factors by binding to an intermediate in the signal transduction pathway, $pp60^{c\text{-}src}$.

iii. Small t antigen

The role of small t protein in polyoma-induced transformation is not yet clear. Noda et al. (1987) showed that small t antigen can cooperate with middle T antigen in transformation. In this study, two distinct vectors were created, one expressing middle T protein, one expressing small t protein. These vectors were transfected simultaneously into NIH-3T3 cells and a strain of normal rat kidney (NRK) cells in culture. The transfectants showed increased growth rate, increased cell density, and increased growth in soft agar compared to those that had been transfected with the middle T-expressing vector alone. These properties, which are associated with the ability of cells to form tumors in test animals, were

not mediated by increased middle T expression or increased protein tyrosine kinase activity. Therefore, it appears that small t codes for a protein that contributes independently to transformation.

b.) SV40

i. Large T antigen

-Structure and function

The 708 amino acid large T protein of SV40 has a complex structure that allows it to carry out a variety of activities during productive infection (regulation of the expression of both early and late viral genes), and transformation (interaction with cellular products). This protein is required for both initiation and maintenance of transformation induced by SV40. Genetic studies with mutated large T genes have identified domains of the large T protein that are sufficient to transform mammalian cells. Clayton et al. (1982) transfected the established fibroblast cell line Rat-1 with a series of recombinant plasmids carrying different portions of the gene encoding large T. A plasmid encoding only the amino terminal half of the protein was capable of transforming the recipient cells to focus formation, but with only 1% of the frequency induced by complete large T protein. Also, the foci induced by the truncated large T protein were morphologically different from those induced by intact large T protein. Other constructs encoding only portions of the amino terminal half of large T protein along with its upstream regulatory sequences could not transform the Rat-1 cells. These investigators concluded that although the amino terminal half alone of

large T protein is sufficient to induce transformation of Rat-1 cells, the transformation probably occurs by a mechanism distinct from that of intact large T protein. In 1987 de Ronde et al. created chimeras of the large T gene from SV40 and BKV (a member of the papova virus family that cannot transform human fibroblasts in vitro as can SV40) to determine the regions of the SV40 large T protein responsible for transformation of cultured human fibroblasts. They showed that the amino and carboxyl terminal halves of SV40 large T protein did not morphologically transform human fibroblasts, but did allow their overgrowth under conditions in which contact inhibition would normally occur. These data support the hypothesis that different domains of SV40 large T induce different transformed phenotypes.

-Mechanism

The mechanism by which large T protein induces transformation is not entirely clear, although several investigators have identified cellular proteins that interact with large T protein. DeCaprio et al. (1988) showed that SV40 large T protein binds the product of the retinoblastoma susceptibility gene, p110-114. Binding occurs at residues 105-114 in the amino terminal half of large T protein. These residues are also required for the transformation by large T protein, supporting the hypothesis that this protein-protein interaction is critical for transformation. This same group of investigators (Ludlow et al., 1989) also showed that large T protein binds to an underphosphorylated form of p110-114, supporting the hypothesis that the underphosphorylated form of p110-114 acts as a tumor suppressor, a function inhibited by large T protein binding. Large T protein also binds the p53 protein (Linzer and Levine 1979), although the

role this plays in transformation remains unresolved. Deppert et al. (1989) studied the effect of two kinds of SV40 large T temperature mutants, one that reverts at non-permissive temperatures (N), and one that does not (A), on the stabilization of p53 protein (an event that is thought to contribute to transformation) and on transformation. They found that at non-permissive temperatures the A (non-reverting) type transformant remained transformed and maintained a stable pool of free p53 protein. However, although large T protein did not form a complex with p53 protein, it did continue to associate with chromatin and the nuclear matrix. In contrast, at non-permissive temperatures, the N (reverting) type transformant reverted, did not maintain a stable pool of p53 protein, and the large T protein could neither bind to p53 protein nor associate with chromatin and nuclear matrix. These investigators concluded that maintenance of transformation requires both large T protein activity and p53 protein stabilization. The transforming region of large T protein has also been shown to bind another human cellular protein, p118 (Ewen et al., 1989), but the normal function of this protein and the importance of its interaction with large T protein to transformation is unknown.

Models currently exist to explain the mechanisms by which large T protein is involved in establishment or immortalization. Mechanisms by which SV40-transformed cells are released from external growth controls are less clear, especially since SV40 does not encode a middle T protein, as does polyoma. In 1988 Khandjian and Gauchat observed that early in abortive infection of mouse cells, SV40 stimulated several rounds of DNA synthesis and mitosis under growth factor-limiting conditions, even though only one round of viral transcription had occurred. This suggests that early in infection SV40 proteins stimulate the growth of the host cell.

It is possible that continued growth occurs in those cells that can integrate and continuously express early viral proteins.

ii. Small t antigen

The role of SV40 small t antigen in transformation is poorly understood. In some cell types the expression of large T protein alone is sufficient to transform, although expression of small t protein often increases the efficiency of transformation. In 1989 de Ronde et al. observed that focus formation is induced by large T protein alone, but that foci of morphologically transformed cells grow on a monolayer only when both large T protein and small t protein are expressed in cells. One explanation for this came from the work of Bikel et al. (1986), who observed that small t protein expression promotes the dissolution of actin cables in the cell. The domain of small t protein shown to be important is a twice-repeated Cys-X-Cys-Cys-X-X-Cys motif. Mutations in this region abolish the ability of small t protein to alter actin cable structure and markedly reduce the efficiency of abortive transformation in Balb/c-3T3 mice infected with this mutant; however, the mechanism behind the correlation between changes in cell architecture and transformation is not known.

Another possible role for small t protein in transformation is in the activation of intracellular metabolic intermediates by phosphorylation. Pallas et al. (1990) purified two peptides of 36kDa and 63kDa that associate with polyoma middle T protein and small t protein, as well as with SV40 small t protein. They identified these as the catalytic and regulatory subunits of protein phosphatase 2A (PP2A), one of the enzymes thought to play an important role in the regulation of metabolism.

Thus, an association between small t protein and PP2A may be another mechanism by which a cell infected with SV40 loses normal growth control.

c.) Papilloma viruses

Papilloma viruses, the third major group classified with the papovavirus family, is comprised of multiple serotypes. Some are associated with benign growths (warts) whereas others are associated with malignant neoplasia. Although the close epidemiological association pointed to a causative role for papilloma viruses in these growths, it was difficult to perform the necessary studies to test this because tight host specificity of these viruses prohibited in vitro propagation of viral cultures. The application of molecular techniques enabled investigators to determine the genomic elements responsible for regulating the viral life cycle, and those involved in transformation. As with the other DNA tumor viruses, the papilloma virus genome is organized into an early region, which encodes regulatory proteins, and a late region, which encodes virion structural proteins. Seven open reading frames or genes (*E1-E7*) have been identified within the early region. Some of these genes, e.g., *E6* and *E7*, overlap and are capable of expressing several products. Of these seven early genes, *E5*, *E6* and *E7* have been shown to be particularly important for malignant transformation. For illustrative purposes, studies relating to two human papilloma virus (HPV) serotypes, i.e., 16 and 18, which are closely associated with human malignancies (zur Hausen, 1987), will be discussed. Baker et al. (1987) showed that the primary viral transcripts in two cervical carcinoma cell lines are derived from the *E6* and *E7* genes. Subsequently, Bedell et al. (1987) constructed plasmids containing the *E6-E7* open reading frame (ORF) from the early

region of HPV 18 and transfected these constructs into NIH-3T3 and Rat-1 cells along with a selectable marker. Selected cells that also expressed the E6 and E7 proteins formed colonies in soft agar, whereas those transfected with either a control plasmid or a plasmid containing a deletion in the *E6-E7* ORF did not. The cells which formed colonies in agar were also tumorigenic in athymic mice. This experiment demonstrated the importance *E6* and/or *E7* to transformation of cultured fibroblasts. Münger et al. (1989) obtained similar results in cultured human keratinocytes.

Although the mechanism by which transformation occurs is not understood, Dyson et al. (1989) showed that the E7 protein from HPV 16 forms a complex with the RB protein p110-114. Since the E7 protein contains a domain with sequence similarity to SV40 large T protein and the E1A proteins of adenovirus (Phelps et al., 1988), both of which are known to complex with the RB protein, an E7-RB protein complex might also be involved in transforming cells. The E6 protein has been shown to bind the p53 protein (Werness et al., 1990), as is also the case for the early region products from SV40 and adenovirus. In fact, studies by Scheffner et al. (1992) show that E6 protein binding promotes the degradation of wild-type p53 protein, whereas some mutant forms of p53 protein do not bind the E6 protein and are not targeted for degradation.

A third open reading frame, *E5*, encodes a small (44 amino acid) protein that is located in the plasma membrane. Although no definitive transforming function has been assigned to HPV *E5*, expression of the E5 product from the closely related bovine papilloma virus (BPV) has been shown to be required for transformation of mouse cells (DiMaio et al., 1986). Green and Lowenstein (1987) showed that the BPV E5 protein can

stimulate DNA synthesis in resting cells. Thus it appears that it is through coordinate expression of a number of viral proteins that papilloma viruses induce cellular transformation.

3. Herpes Viruses

The group of Herpes viruses that can infect humans is comprised of *Herpes simplex* virus (HSV) 1, 2, and 6, cytomegalovirus (CMV), *Varicella zoster* virus (VZV), and Epstein-Barr virus (EBV). Each has a complex genome that can take several isomeric forms. Typically, infection with the virus results in lysis of the affected cell; however, a latent infection can be established in which no virion production is observed, but low levels of some viral protein products are detected. By mechanisms that are not well understood, the latent virus can be reactivated to a lytic cycle. During reactivation the affected individual has clinically apparent symptoms. Several factors, including the ongoing work to determine the role of other DNA viruses in cellular transformation, the knowledge that members of the herpes family are capable of establishing latent infection in mammalian cells, and the proposed link between *Herpes simplex* and cervical cancer (Manservigi et al., 1986), led researchers to look for transforming regions in the genome of herpes viruses. Studies were complicated by the lack of non-permissive cells in which infection would occur without lysis. Even in cells infected with defective (non-lytic) virus, little of the viral genome remained, and that which did showed only limited sequence similarity to other herpes viruses, or to known transforming regions of the DNA tumor viruses. Transformation associated with Herpes infection will be discussed using Epstein-Barr virus as an example.

Of all the Herpes family, the clearest epidemiological link between infection and transformation is demonstrated by the relationship observed between EBV infection and Burkitt's lymphoma or nasopharyngeal carcinoma. EBV is also capable of immortalizing B lymphocytes in culture and of transforming established rodent cell lines. Two viral products have been determined to be responsible for transformation. These are Epstein-Barr virus nuclear antigen 2 (EBNA-2) and latent infection membrane protein (LMP). Dambaugh et al. (1986) transfected into NIH-3T3 and Rat-1 cells a series of expression vectors, carrying both a selectable marker and various regions of the EBV genome. Rat-1 cells transfected with a vector containing EBNA-2 grew better in low serum medium than cells transfected with the selectable marker alone. Ability to grow in low serum is a property commonly found in malignant cells. To elucidate the mechanism by which this growth advantage occurs, Wang et al. (1987) infected an EBV-negative Burkitt's lymphoma cell line with retroviral constructs containing genes coding for each of the 4 EBNA proteins, the EBV leader protein, or the EBV LMP and observed changes in the expression of B cell surface markers and growth. They found that the EBNA-2-containing construct stimulated the expression of CD23, a surface marker of B cell activation that is thought to act by transducing the B cell growth factor signal. Calender et al. (1987) found no induction of the expression of this marker, nor of immortalization, in a strain of EBV that does not produce a functional EBNA-2. Wang et al. (1985) demonstrated that expression of the LMP in established rodent cells in culture results in transformation. NIH-3T3 cells expressing the LMP were morphologically altered, and LMP-expressing Rat-1 cells formed foci, colonies in soft agar, and progressively growing tumors in athymic mice.

Interestingly, latent EBV infection is common in the United States, but there is no obvious association with an increased incidence of either Burkitt's lymphoma or nasopharyngeal carcinoma. Given the multi-stepped nature of malignant transformation, it seems likely that other genetic and/or environmental factors play a role in this disparity in clinical outcome based on geography.

The knowledge obtained about the biology of this class of viruses has provided valuable insights into the mechanisms by which they cause disease. In the process, data have accumulated that support the notion that malignant transformation is a multi-stepped process. Furthermore, increased understanding of the interaction between viral and cellular products has laid the groundwork for a more general understanding of cellular transformation.

B. Retroviruses

The retroviral life cycle consists of infection of the host cell, synthesis of a complementary double-stranded DNA provirus from viral genomic RNA using reverse transcriptase encoded by the virus, and integration of the provirus into the host genome, followed by transcription, packaging and release of viral progeny. Retroviruses are considered to transform human and animal cells (reviewed by Bishop, 1983), and this transformation is thought to be effected by the same mechanisms that occur in the viral life cycle. One mechanism involves transduction and transfer of a cellular gene, for example, *src*, *ras*, and *sis*, to a new cell in which it is inappropriately expressed and/or exists in mutated form. A second mechanism is insertion of the provirus near to a gene whose expression is normally tightly regulated. This results in

inappropriate expression of that gene (e.g., *myc*, and *myb*). A third mechanism involves insertion of the provirus in such a position that it results in production of a chimeric protein consisting of both proviral and endogenous elements (e.g., *erbB*). A fourth mechanism is inactivation of an endogenous gene by insertion of proviral DNA into the gene itself. Examples include Friend spleen focus-forming virus (FSSV) and *p53*. Still another mechanism consists of regulatory elements within the viral genome that act to alter the expression of host genes (e.g., trans-activation by *tat* of HTLV, HIV). Understanding these mechanisms will provide insight not only into the biology of this virus family, but also into parallel endogenous cellular processes causing malignant transformation.

1. Transduction of an Activated Gene

Doolittle et al. (1983) first proposed that the simian sarcoma virus (SSV) transforming protein $p28^{v\text{-}sis}$ is encoded by the transduced form of the human gene encoding platelet-derived growth factor B chain (PDGF-B) based on finding a high degree of similarity between the two proteins. This was an important discovery in light of prevailing knowledge that certain viruses had transforming capacity. Infection by a virus known to carry a cellular gene encoding a protein growth factor for mesenchymal cells provided a plausible explanation for the induction of sarcomas. Subsequent studies by Fry et al. (1986) established that insertion of v-*sis* into normal diploid human fibroblasts results in transformation as measured by focus formation and anchorage independence. However, the transformants do not acquire increased life span, nor are they tumorigenic in athymic mice. Pech et al. (1989) was able to induce fibrosarcomas in newborn mice by infection with a c-*sis*-containing retroviral vector that

overexpressed PDGF-B. However, these fibrosarcomas were polyclonal rather than monoclonal, which supports the hypothesis that overexpression of PDGF-B is only one of several changes that together result in malignant transformation.

Several studies were performed by Aaronson and co-workers to determine the sequences in v-*sis* that are critical for transformation. The central region of $p28^{v\text{-}sis}$ shows the highest level of sequence similarity to PDGF-B. Giese et al. (1987) showed that eight cysteine residues exist within the central region of $p28^{v\text{-}sis}$. Of these eight residues, four could not be altered without loss of transforming function. The Cys residues in the central region are responsible for correct formation of intra-chain disulfide bonds thought to be critical for PDGF receptor recognition. Other work on this central region (Giese et al., 1990) demonstrated that these Cys residues do not actually lie within the minimal transforming region of $p28^{v\text{-}sis}$ (i.e., the receptor binding domain), leading to the hypothesis that correct tertiary structure is required for function of PDGF-B. The minimal transforming domain was also shown by LaRochelle et al. (1990) to contain recognition specificity for the beta-type PDGF receptor.

The structure/function relationships established by these investigations led to hypotheses regarding mechanisms by which PDGF-B stimulates transformation. Of specific interest was whether PDGF-B needs to be expressed at the cell surface to interact with its receptor. To answer this question, studies were performed to determine the correlation between post-translational processing/transport of PDGF-B and its transforming ability. Hannick and Donoghue (1986) constructed vectors containing the gene coding for the membrane anchor domain of a G-protein

fused to v-*sis*. They found that formation of correct quaternary structure (i.e., dimerization and glycosylation) is associated with transport of the protein product to the cell surface and that transport to the cell surface correlates with transforming ability. They concluded that transport to, and activity at, the cell surface is important for transformation by the v-*sis* product; however, they could not extrapolate the results in their experimental system to endogenous PDGF.

Although there was little doubt that interaction between PDGF-B and its receptor was required for mitogenesis and transformation, debate continued about the location of this interaction. Data from Huang et al. (1984), showing that antibodies to PDGF-B could only partially inhibit transformation by SSV, led to the hypothesis that there are both extra- and intra-cellular locations for PDGF-receptor interactions. In subsequent work, Huang and Huang (1988) also studied the kinetics of PDGF receptor (PDGFR) processing and turnover in NIH-3T3 and normal rat kidney (NRK) cells in the presence and absence of PDGF. Their data indicated that few newly synthesized PDGFR molecules are transported to the cell surface prior to degradation. Moreover, suramin, which inhibits both cell surface and cytoplasmic PDGF-receptor interaction, altered the PDGFR turnover, whereas protamine, which acts at the cell surface only, did not. They concluded that *sis* transformation occurs by an intracellular autocrine loop. This conclusion was supported by Bejcek et al. (1989) who transfected NRK cells with a v-*sis* mutant targeted to other endoplasmic reticulum (ER) and golgi. The transfected cells formed foci, and cell fractions from ER and golgi expressed v-*sis* product that was mitogenically active, but no *sis* product was detected outside the cells.

Although the data supporting an intracellular autocrine loop

mechanism of transformation by *sis* are strong, the fact that anti-PDGF antibodies can inhibit transformation partially suggests that an extracellular component also exists. Lokeshwar et al. (1990) compared the kinetics of PDGF-B turnover and mitogenic activity. They showed that the mature PDGF-B species (a 44kDa protein) interacts with some molecule other than PDGFR on the cell surface, since treatment with suramin, which inhibits the binding of growth factors to their receptors, released free p44, whereas none was released by treatment with a specific PDGF receptor-binding inhibitor, protamine. Definitive determination of the mechanism (i.e., internal vs. external autocrine loop) by which *sis* activity results in mitogenesis and transformation does not yet exist. However, these studies have provided insight into the mechanisms of transformation mediated by expression of protein growth factors, and into one of the means by which retroviruses are capable of inducing transformation.

2. Insertional Activation

The viral oncogenes *myc* and *myb* exemplify transformation by retroviral insertional activation. The products of these cellular genes have been shown to be located in the nucleus (*myc* by Donner et al., 1982; Alitalo et al., 1983; *myb* by Klempnauer et al., 1984) and to bind to DNA. Therefore, they are thought to act as transcriptional activators. In sequence comparison studies, Ralston and Bishop (1983) found regions of sequence similarity between *myc*, *myb*, and *E1A* of adenovirus. Overall sequence identity is 15-21%, but alignment studies showed clustered regions of increased similarity. Hydrophobicity profiles are also alike, further supporting the hypothesis that these proteins have a similar function in the cell. One possibility is that they, like the E1A

proteins, act by releasing the cell from limited life span.

In 1977, Bister et al. showed that the transforming region of the acute avian leukemia virus MC29 encodes a *gag* fusion product later found to be *gag-myc*. A similar product was identified as part of the defective viral particle released by chicken myeloblast cells transformed without helper virus (Duesberg et al., 1980). This product did not contain *myc*, but rather the related *myb* gene fused with the viral *gag* and *pol* genes. In 1981, Hayward et al. hypothesized that the slow and the acutely transforming retroviruses transform cells using different mechanisms. Specifically, the slow acting viruses transform by integration into the host genome, causing inappropriate expression of endogenous host genes, whereas the acutely transforming viruses transform by inserting an exogenous transforming gene. These investigators based their hypothesis in part on the observations that protein-encoding segments of viral genome are often absent in infected cells. They screened several lymphomas and one lymphoma-derived cell line for the presence of oncogene mRNA, and found a significant increase in the expression of *myc* mRNA. This increase in expression was correlated with the expression of sequences corresponding to the five- and three-prime ends of the viral genome (i.e. the LTR) and not of the viral structural genes (*gag*, *env*, and *pol*). Also, restriction enzyme digestion and subsequent analysis of *myc* and its flanking sequences showed that the provirus had integrated near to the endogenous *myc* gene. Hayward et al. (1981) concluded that insertional activation of *myc* is a common mechanism by which the slow-acting viruses transform their host cell.

Data by Gauwerky et al. (1988) illustrate the applicability of the retroviral insertional activation model to the understanding of the

process of malignant transformation mediated by endogenous *myc*. They identified rearrangements involving activation of the *myc* gene that were associated with progression of malignancy in patients with pre-B-cell leukemia. They did not report data on changes in *myc* expression in the cells with rearrangements. Future experiments linking rearrangements in *myc* in both viral and endogenous systems with changes in expression will be important to gaining a clearer understanding of the importance of this mechanism in malignant transformation.

3. Alteration of a Gene Product

The oncogene v-*erbB*, isolated from avian erythroblastosis virus (AEV) is structurally similar to the class of viral oncogenes that includes *src*, *fes* and *abl*. These genes encode protein tyrosine kinases that presumably regulate cell growth and differentiation. Because of the known association between viral oncogenes and their cellular counterparts, Downward et al. (1984) compared the sequence of purified epidermal growth factor receptor (EGFR), which is also known to have tyrosine kinase activity, with that of the product of the v-*erbB* gene. The EGFR has been divided into three domains, an extracellular domain that binds the ligand (EGF), a transmembrane domain, and an intracellular domain that has both tyrosine kinase activity and tyrosine residues that are sites for phosphorylation. These investigators found high sequence identity between the v-*erbB* protein and portions of EGFR; however a striking difference was the lack of the extracellular ligand binding domain in the v-*erbB* protein. Other alterations in v-*erbB* include a deletion in the carboxyl terminus-encoding region, a small internal deletion in the intracytoplasmic domain-encoding region, and several point mutations.

The regions of v-*erbB* that are vital to its transforming ability were determined by Wells and Bishop (1988). They created chimeric genes by substituting regions of v-*erbB* with corresponding fragments from the EGFR gene, c-*erbB1*. They found that loss of sequences encoding the amino terminus (ligand binding domain) is necessary and sufficient to cause malignant transformation (tumorigenesis) in Rat-1 and NIH-3T3 cells. Neither the carboxyl region-encoding deletions nor point mutations present in v-*erbB* were found to be required for transformation, although these do increase the relative transforming efficiency and alter the host range of the constructs.

Sequence and gene expression data from several AEV strains revealed the presence of retroviral *gag* and *env* genes in close proximity to the transduced *erbB* gene. Bishop and coworkers (Bruskin et al., 1990) found that the product of the v-*erbB* gene is a fusion protein containing several residues from the nearby *gag* gene. Vectors constructed to determine the effect of those *gag* residues on transformation showed that the presence of six *gag* amino acids resulted in a hundred-fold increase in transforming activity in NIH-3T3 cells. The increase in transformation was not accompanied by increased expression of the fusion product. They speculated that the increase in transforming efficiency is the result of alterations in the post-translational processing of the fusion protein. Similar studies by Maihle et al. (1988) had shown that insertional activation of c-*erbB* by avian leukosis virus (ALV) results in various *erbB* transcripts, including those coding for *gag-erb* and *gag-env-erb* fusion products. Vectors mimicking these fusion products were processed differently by the host cell, resulting in proteins with unique glycosylation patterns and cellular localization. Interestingly, those

products that were encoded by *env*-minus constructs were less likely to be found at the cell surface. They speculated that such differences in post-translational processing and subsequent cell localization have an effect on the transforming ability of fusion proteins. Lee and Hayman (1990) also discovered the importance of the *gag-erb* fusion product in experiments designed to test the role of the extracellular domain of v-*erbB* in transformation of chicken fibroblasts and erythroblasts. A vector was constructed to use the *env* gene to localize the protein to the cell membrane. However, the only stable transfectants found contained a recombinant *gag-env-erbB* fusion product, leading to the speculation that one function of the extra-membrane and trans-membrane domains of c-*erbB* is the localization of the product to the appropriate cellular location. In the case of v-*erbB* this function would be assumed by the viral *gag* and *env* genes.

Early studies involving the intracytoplasmic domain of v-*erbB* had shown that alterations in the tyrosine residues typically available for phosphorylation did not abolish transforming capability, but did change the transforming efficiency and/or altered the host cell specificity for transformation. Raines et al. (1988) identified three viral strains that differed in erbB structure, specifically in the arrangement of tyrosine residues. One strain was only capable of inducing erythroblastosis; another induced both sarcomas and hemangiomas; and the third induced erythroblastosis and sarcomas. The cause of strain-specific cell type transformation was not clearly defined; however, the authors hypothesized that the erbB products produced by the three strains interact with downstream effectors unique to each cell type(s). They reasoned that further experiments to determine the substrates of the erbB protein

tyrosine kinase might yield insights into the mechanism of transformation in different cell types. Aaronson and colleagues (Di Fiore et al. 1990) compared the transforming capacity of c-*erbB1* and c-*erbB2* in NIH-3T3 cells and 32D cells, a mouse hematopoietic cell line lacking both c-*erbB1* and c-*erbB2*, and typically dependent on IL-3 for proliferation. c-*erbB2* is an altered form of c-*erbB* that is associated with malignant transformation of human cells, and has been shown to be amplified in mammary carcinoma and salivary gland carcinoma. Two 32D strains were established after transfection. One expressed high levels of the c-erbB1 protein, the other expressed high levels of the c-erbB2 protein. However, the c-erbB1 protein was only phosphorylated when the cells were grown in the presence of EGF, whereas c-*erbB2* transformants had a high endogenous level of c-erbB2 protein phosphorylation even without EGF. However, even when c-*erbB2* was highly expressed and phosphorylated, IL-3 was required for anchorage independence, suggesting that the c-erbB2 product is not capable of efficient interaction with downstream regulators of anchorage independence. NIH-3T3 cells transfected only with c-*erbB1* could be efficiently induced to anchorage independence with EGF stimulation, indicating that the c-erbB1 protein effectively interacts with other growth-regulating elements in these cells.

Upregulation of the tyrosine kinase activity of the erbB protein has also been shown to correlate with increased transforming efficiency. Segatto et al. (1988) generated mutants of c-*erbB2* that were upregulated by association with an LTR or the SV40 promoter, encoded a protein with amino terminal deletions, or else with a point mutation at codon 659 (the codon shown to be altered in the mouse equivalent of the c-*erbB* gene). In each case, in vitro tyrosine kinase activity of the altered c-erbB product

correlated with transforming efficiency in NIH-3T3 cells. In view of their data, the authors suggested using tyrosine kinase activity as a screening tool for *erbB* mutations. They also speculated that further studies on cells that have increased tyrosine kinase activity would yield insights into the substrates for the tyrosine kinase that serve as downstream regulatory points for growth and differentiation.

4. Insertional Inactivation

Another mechanism by which retroviral oncogenes act is inactivation of an endogenous gene. An example of this is the inactivation of the p53 protein by gene rearrangement in cells transformed with the Friend spleen focus forming viruses (FSSV). Monroe et al. (1988) reported the presence of a truncated form of the p53 protein in several FSSV-induced erythroleukemic cell lines. This altered 46kDa protein is formed by an in-frame deletion of one of the highly conserved regions of *p53*, and was shown to be incapable of binding with SV40 large T antigen. Two other rearrangements involving *p53* were reported by Ben David et al. (1988). One is an internal deletion with a frameshift and the other was insertion of the viral genome into the *p53* locus. Because of the presumed tumor suppressor function of the p53 protein, loss of a functional product as a result of retroviral infection is expected to contribute directly to the malignant transformation of the infected cells.

5. Trans-Activation of Cellular Genes

Most retroviruses have been shown to transform through cis-acting mechanisms. With the advent of research into the lymphotrophic viruses, i.e., human T cell lymphotrophic virus (HTLV) and human immunodeficiency

virus (HIV), trans-acting transforming elements have been discovered. Nerenberg et al. (1987) showed that the expression of the tat protein (known to be a trans-acting element in the HTLV I genome) is highly associated with malignant transformation. They created transgenic mice carrying the *tat* gene flanked by a viral LTR and poly A site. This construct had previously been shown to result in the expression of a correct product that induces the expression of a reporter gene. When the *tat* gene was expressed in muscle, soft tissue sarcomas formed. Gene expression was also seen in the thymus; however, in these mice thymic atrophy and death occurred. Further studies by this group (Vogel et al., 1988) used the HIV *tat* gene introduced into transgenic mice. In this case expression of the HIV *tat* was seen only in the skin, and Kaposi sarcoma-like tumors formed in most of the male transgenic animals. Interestingly, no transgenic females were affected. However, when the cells from the Kaposi sarcoma-like tumors were examined, expression of the *tat* gene was not observed. One explanation for this phenomenon is that *tat* expression is required early in the process of transformation, but is not needed at later stages. Another possibility is that the tat product activates the expression of regulatory genes (e.g. growth factors) in the cells adjoining the transformed cell, and thus has an indirect transforming effect. Further studies will be needed to determine the importance of trans-activation in malignant transformation.

C. Transformation by Oncogenes:

Genetic alterations play an important role in malignant transformation. These alterations fall into two major categories, activation of dominant-acting proto-oncogenes, and inactivation of tumor

suppressor function requiring the loss of both alleles encoding that suppressor. An overview of current understanding of both these categories will be briefly presented with examples.

1. Dominant-Acting Oncogenes

Activation of proto-oncogenes can occur by point mutations that lead to altered protein function, or by changes that lead to unregulated protein expression. Approximately forty such genes have been found, each encoding a protein critical to the regulation of growth and differentiation.

Oncogene products are found at different levels of cellular growth pathways, namely membrane-associated proteins (growth factors and their receptors, membrane-bound signal transduction proteins, and protein kinases), cytosolic proteins (protein kinases, modulatory proteins), and nuclear proteins (DNA-binding proteins, transcriptional activators). Much of what is known about dominant-acting oncogenes has resulted from investigations into the transforming retroviruses, as discussed in the previous section. Because the acutely transforming retroviruses carry genes derived from endogenous cellular genes that have been altered during the course of their incorporation into the viral genome, and/or as viral genes evolved, they provide clues to how their endogenous counterparts act.

a.) Genes encoding membrane-associated proteins

The prototype for understanding the relationship of viral-encoded transforming factors and their cellular counterparts is the *src* gene. The virus that transforms chicken fibroblasts contains the *src* gene, first

described by Rous in 1911 (reviewed in Seemayer and Cavenee, 1989). The *src* gene encodes a 60kDa protein that associates with the cell membrane, has tyrosine kinase activity, and can be regulated by phosphorylation of tyrosine residues within its sequence. The v-*src* gene has been shown to have an endogenous cellular counterpart in several species. In 1983 Takeya and Hanufsa reported the sequence of the v-*src* homologue in chickens, and compared the sequence and structure to that of v-*src*. They found that the amino acid sequences vary at only 28 positions. The structure and sequence of c-*src* has been shown to be highly conserved among diverse species, suggesting that *src* plays a critical role in the normal function of the cell. In a comparison between human and chicken c-*src*, Anderson et al. (1985) showed a DNA sequence identity of approximately 90% and a amino acid sequence identity of approximately 98%.

The mechanism by which proto-*src* becomes activated has been the subject of much ongoing research. The wild-type protein is known to be membrane bound, and to have low-level tyrosine kinase activity. Iba et al. (1985) compared several properties of v-*src* and c-*src* and found no difference in protein expression level or subcellular location. They did show that $pp60^{v\text{-}src}$ has a much higher tyrosine kinase activity, and that the level of kinase activity correlates well with transforming capacity. In similar studies, Johnson et al. (1985) constructed plasmids containing v-*src* and c-*src* under the control of strong promoters to confirm that over-expression of the $pp60^{c\text{-}src}$ is insufficient to transform cells. They isolated NIH-3T3 c-*src* and v-*src* transfectants either by focus formation or by selection for a co-transfected selectable marker. In a few cases focus-forming, non-tumorigenic c-*src* transformants were found, and all of these expressed $pp60^{c\text{-}src}$ at five times the level of $pp60^{v\text{-}src}$. Even at these

high levels of expression, the kinase activity in the c-*src* transfectants did not reach that of the v-*src* transfectants. This supported the hypothesis that increased $pp60^{src}$ tyrosine kinase activity is required for transformation. Coussens et al. (1985) showed that $pp60^{c\text{-}src}$ and $pp60^{v\text{-}src}$ could be distinguished not only by tyrosine kinase activity, but also by the pattern of proteins phosphorylated in vivo, suggesting that transformation by v-*src* involves subverting normal metabolic pathways to cause abnormal growth. Clearer understanding of transformation by activation of *src* awaits data regarding normal substrates for this tyrosine kinase, and their function in regulating cell growth.

b.) Genes encoding nuclear proteins

Oncogene products found in the nucleus and thought to act by regulating gene expression are exemplified by c-*jun*. This oncogene was first identified as the transforming agent in an avian sarcoma virus (ASV-17). Cellular homologues have been identified in chicken, mouse and human cells. Researchers found significant (44%) sequence identity between v-jun product and the yeast transcription activator GCN4. This transcription activator is similar to the cellular protein AP-1 in amino acid sequence and DNA binding specificity (reviewed by Curran and Franza, 1988). Bohmann et al. (1987) performed several experiments that provided evidence that the c-jun protein and AP-1 are related. First, antibodies raised against the v-jun protein cross-reacted with AP-1. Second, the v-jun and c-jun proteins had an overall sequence identity of 80%, with greatest similarity (only 2 amino acid changes) in the carboxyl terminal domain known to be responsible for DNA binding. Sequence similarity also held across several distantly-related species. Third, trypsin digest

fragments of AP-1 were easily matched with the putative sequence of the c-jun protein. Fourth, DNA binding footprints were essentially identical for purified AP-1 and for the protein product encoded by a vector encoding the carboxyl terminal 133 codons of c-jun. It was noted; however, that even purified AP-1 had several different MW species, leaving open the possibility that there is a family of DNA binding proteins with similar binding specificity. Some support for this hypothesis was obtained by finding more than one band when probing genomic DNA with v-*jun* under low-stringency conditions. Rauscher et al. (1988) identified a nuclear protein p39 as being AP-1, and also as the product of the c-*jun* gene. This protein is one of several that associate with another nuclear oncoprotein $p62^{c\text{-}\underline{fos}}$. AP-1 and p39 extracts were shown to co-migrate, as does p39 that has been immunoprecipitated by antibodies to $p62^{c\text{-}\underline{fos}}$. Trypsin digestion fragments of p39 are identical to the protein produced by a vector encoding the c-jun protein. Sequential immunoprecipitation experiments also showed protein-protein interaction between the products of *fos* and *jun*.

Lamph et al. (1988) explored the functional role of c-*jun* in mouse NIH-3T3 cells. They investigated the effect on the expression of mouse c-*jun* of treatment with serum and TPA, both of which are known to stimulate cell growth and to induce expression of *fos*. Expression of jun product was induced by both serum and TPA, and the time course of induction was very similar to that of induction of *fos*. The product encoded by their construct was also capable of <u>trans</u>-activation of a reporter gene, chloramphenicol acetyl transferase (CAT). Schütte et al. (1989) tested the ability of wild-type, but deregulated, human c-*jun* to transform rat cells. Vectors were constructed that contained genomic c-*jun* and c-*jun*

cDNA in both sense and antisense orientation. These were transfected into primary rat embryo cells and the immortal rat fibroblast cell line Rat 1. The Rat 1 line was transformed to anchorage independence and tumorigenicity when c-*jun* mRNA was highly expressed, i.e. at levels 10-15 times more that of endogenous *jun* mRNA. Primary rat cells could not be transformed by transfection of *jun* alone or by transfection of c-*jun* in combination with c-*myc*. However, when *jun* was co-transfected with activated H-*ras* gene, the transfectants showed increased focus formation. These focus-forming cells were capable of producing tumors in athymic mice. Transforming efficiency was augmented further by treatment of transfected rat embryo cells with the phorbol ester TPA.

These studies highlight the complexity of pathways controlling cell growth and differentiation, and establish the importance of activation of dominant-acting proto-oncogenes in transformation of mammalian cells. Transformation induced by the activation of another family of genes, the *ras* genes will be discussed in subsequent sections of this review.

2. Tumor Suppressor Genes:

The other general genetic mechanism contributing to the malignant transformation of cells is the inactivation of tumor suppressor genes. This class of genes, which includes the retinoblastoma susceptibility gene (*RB*), the *p53* gene, the Wilms' tumor sensitivity gene, and the gene associated with the development of colorectal carcinoma (*MDM2*), has been studied extensively. However, as they are not directly related to the research topic of this thesis, consideration of their properties will be limited to a brief discussion of the *RB* and *p53* genes (reviewed by Levine and Momand, 1990).

a.) The retinoblastoma susceptibility gene

Loss of both alleles of the *RB* gene was first postulated by Knudson in 1971 after an examination of epidemiological patterns of retinoblastoma occurrence. Retinoblastoma is found in two distinct patterns, the first being the early appearance (i.e., average age 14 months) of bilateral retinal tumors in patients with a family history of retinoblastoma, the second being the later appearance (i.e., average age 30 months) of unilateral tumors in patients with no family history of this disease. Knudson hypothesized that in the first case, patients inherit a germ-line mutation that increases their risk of acquiring a second change in one of the altered retinal cells. This "two hit" hypothesis was substantiated when the Rb gene was localized to chromosome 13 (Yunis and Ramsay, 1978) and cloned (Lee et al., 1987). Restriction fragment length polymorphism (RFLP) analysis of DNA from retinoblastoma cells showed the loss of both alleles of the *RB* gene (Cavenee et al., 1983; Friend et al., 1986). Furthermore, in vitro data showed that returning one wild-type allele to retinoblastoma cells reverses the malignant phenotype (Huang et al., 1988). This suggested that wild-type *RB* is somehow involved in the regulation of cell growth. Further studies have shown interactions between the product of the *RB* gene and several of the transforming proteins of the DNA tumor viruses (Whyte et al., 1988; Larose et al., 1991 DeCaprio et al., 1988; Phelps et al., 1988), implicating the inactivation of the tumor suppressor function as the mechanism of transformation by these viruses.

b. The *p53* gene

The *p53* gene has been localized to the short arm of chromosome 17 (Benchimol et al., 1985; Isobe et al., 1986). Loss of activity of its product has been implicated in a variety of tumors. However, an understanding of the role of *p53* in malignant transformation has been complicated. Early experiments by Rotter (1983) showing the overexpression of the p53 protein in several transformed mouse cell lines, as well as data showing that mutated *p53* can cooperate with other oncogenes in transformation (Hinds et al., 1989), led to speculation that *p53* acts similarly to the dominant oncogenes. In contrast, studies exemplified by Finlay et al. (1989), have shown that expression of wild-type p53 protein suppresses transformation induced by *ras* and adenovirus *E1A* or by *ras* and mutant *p53* in rat embryo fibroblasts. Additional investigation into the properties of mutant p53 protein have shown that it has an altered conformation (Finlay et al., 1988), altered oligomer formation (Kraiss et al., 1988; Milner et al., 1991; Stenger et al., 1992), altered protein-protein interaction (e.g., binding the heat shock protein encoded by *hsc70* as shown by Finlay et al., 1988), and an altered half life (Finlay et al., 1988). These data support the hypothesis that mutant p53 protein subunits, when interacting with wild-type p53 protein subunits, inactivate the function of the wild-type protein in the transformed cell (Eliyahu et al., 1988). This hypothesis is consistent with a model in which the normal tumor-suppressing function of p53 protein is lost during malignant transformation. Further support that loss of normal p53 protein function is required for the transformation of some cells comes from data showing that the transforming proteins of the DNA tumor viruses interact with p53 (Linzer and Levine, 1979; Sarnow et al.,

1982; Werness et al., 1990), and that the Friend leukemia virus can inactivate *p53* (Ben David et al., 1988).

Future studies of this class of genes will undoubtedly lead to significant insights into the normal regulation of cell growth and differentiation, as well as the derangement of these processes in the course of malignant transformation.

III. Epigenetic Contributions to Malignant Transformation

Early contributions to understanding malignant transformation were based on genetic models supported by experiments showing that transformation is induced by the acutely transforming RNA tumor viruses. The transforming agents in these viruses were shown to be transduced cellular genes, as discussed in earlier sections of this review. As evidence accumulated supporting the multi-stepped theory of transformation, it became clear that not all of the changes occurring in malignant transformation were necessarily genomic alterations. Specifically, tumor promoters seemed to work by a different mechanism. As knowledge of malignant transformation has developed, theories have been advanced to explain in vivo and in vitro data that call into question the universality of mutation-induced carcinogenesis.

A. Developmental and Environmental Theories

In 1985, Rubin criticized theories of carcinogenesis that included only genetic events. In his view, even the inclusion of epigenetic phenomena such as DNA methylation was insufficient to account for the complexity of the transformation process. He maintained that consideration of developmental issues and cellular organization is

required in order to account for several experimental findings in the field of carcinogenesis. First, he cited data showing that transplanted stem cells either differentiate or become transformed depending on the area to which they were transplanted, as well as studies describing tumor heterogeneity. He claimed that these data could not be explained by a mutation-based model, but rather that they supported the view that the phenotype of cells depends upon the surrounding environment, sometimes referred to as "field effects". Second, he discussed studies showing the progressive nature of transformation. Some early-passage cells taken from tumors have a non-metastatic, slow-growing phenotype and near-diploid genotype, but with time and serial passage in culture, cells become metastatic, aggressively-growing, and karyotypically more abnormal. He interpreted this as evidence that the tumor cells respond to a particular environment, either in culture or in a test animal. Finally, he cited experiments showing that cells are differentially susceptible to chemically induced transformation depending on their surrounding environment, (e.g. within an organ system or in monolayer culture), and sparse or dense distribution in a culture dish. His explanation for these data was that the surrounding environment in which a cell exists is critical to its behavior, and in particular its transformability.

Although it is important to evaluate results carefully in the context of an experimental system, the findings Rubin cited to refute the importance of genetic phenomena to transformation can, in fact, be explained by genetic mechanisms. In each case, there are known genetic mechanisms that would allow cells in the surrounding environment to modulate gene expression within the target cell. Such pathways include growth factors and their receptors, second-messenger systems, basement

membrane-protease interactions, alteration of gene expression by the action of nuclear transcription factors, and activity of tumor suppression factors. Selective conditions would allow for expansion of a specific cell type and/or genetic change. Similarly, altered regulation followed by subsequent rounds of selection would result in progressive changes in cell phenotype.

B. Methylation

One area of epigenetic control of cell function thought to play a role in transformation is DNA methylation-mediated alteration of gene expression. In a review of this subject, Holliday (1987) first made a case for the heritability of methylation patterns, as heritability is an important consideration in multi-step carcinogenesis. Primary evidence included data showing that the X chromosome that is inactivated in each generation can be traced stably to either maternal or paternal origin. This process is called chromosomal imprinting. Second, there is an increased incidence of the development of tumors in the offspring of animals exposed to carcinogenic agents. The rate of mutation required to account for this increase is unreasonably high. Another possible explanation is a heritable alteration in methylation patterns which would predispose to tumor formation. One example that suggests a role for methylation in transformation is the interspecies difference in the rate of carcinogenesis. Rates of cancer development in rodents are much greater than that of humans, even though their cells can be shown to have similar mutation rates. Holliday argues that differential maintenance of methylation explains the difference. Although our knowledge of the mechanisms by which methylation is maintained and/or altered has increased

markedly since 1987, specific mechanisms by which methylation regulates gene expression still remain to be elucidated. Further studies are needed to understand how normal cellular processes are controlled by DNA methylation, and the role that methylation plays in the malignant transformation of cells.

C. Protein Kinase C

Alteration in second-messenger systems as a result of protein kinase C (PKC) activity is another example of an epigenetic phenomenon that can play a role in the process of transformation (c.f., review by Weinstein, 1991). Weinstein emphasizes the importance of epigenetic changes in malignant transformation, given that-non-genotoxic chemicals are known to be involved in the induction of transformation, and that the behavior of transformed cells can be modulated by non-genotoxic agents (e.g. hormones). He considers that since normal growth and differentiation of cells is presumably regulated by both genetic and epigenetic factors, one can postulate that alterations in both types of factors are required for malignant transformation. Data from experiments performed in his laboratory showed that a high expression level of PKC in rat fibroblasts is associated with an increased susceptibility to transformation by the T24 H-*ras* oncogene (Hsiao et al., 1989), and that overexpression of PKC in cultured rat liver epithelial cells results in altered expression of the myc protein, phorbin, and ornithine decarboxylase (Hsieh et al., 1989). He and co-workers also performed studies implicating alterations of PKC activity in the transformation of colon epithelium (Guillem et al., 1987).

The concept of malignant transformation as a multi-stepped process resulting from alterations in the complex pathways controlling normal

cellular growth and development has been well documented. One general approach to gaining insights into these processes is to consider the cellular processes which are altered in the process of transformation. Attempts to define the likely mechanisms for these alterations should involve investigations of both genetic and epigenetic phenomena. The explosion of data in the field of oncogenes has certainly contributed to the understanding of transformation. Similar investigations into the role of epigenetic phenomena in malignant transformation might also yield significant insights.

IV. *ras* Proto-Oncogenes and Malignant Transformation

A. General Properties of the *ras* Family

1. *ras* Family

The *ras* proto-oncogenes are a family of genes within the superfamily of genes encoding guanine nucleotide-binding proteins. These genes encode proteins involved in many aspects of cell growth and regulation (reviewed by Valencia et al., 1991). H-, K- and N-*ras* are the most widely studied in this family because they have been implicated in the process of malignant transformation since the discovery of the Harvey rat sarcoma virus and Kirsten sarcoma virus. However, the identity and functions of the protein products of other members in this gene family have recently become clearer. For example, sharing approximately 30% sequence identity to the *ras* genes are the genes of the Rho family and the Rab family. The former have been implicated in maintenance of the cytoskeleton, and the latter appear to be involved in the regulation of secretory functions

(reviewed by Hall, 1990).

2. Structural Properties of *ras*

a.) DNA

The *ras* genes were discovered when the transforming agent in the Harvey Murine sarcoma virus was identified (Harvey, 1964). A similar gene was identified in the Kirsten Murine sarcoma virus (Kirsten and Mayer, 1967). Later these genes were found to have human cellular homologues (Parada et al., 1982; Ellis et al., 1981; McGrath et al., 1983). N-*ras* has been identified within a human neural cell tumor (Shimizu et al., 1983) and human sarcomas (Hall et al., 1983), and has not been shown to have a viral counterpart. H-*ras* is located on chromosome 11 (McBride et al., 1982; Popescu et al., 1985), K-*ras* on chromosome 12 (Popescu et al., 1985), and N-*ras* on chromosome 1 (Popescu et al., 1985). In addition, in human cells there are processed pseudogenes for H- and K-*ras* that are not expressed and are thought to have arisen by insertion of a processed mRNA into the germline (McGrath et al., 1983; Miyoshi et al., 1984).

The general structure of the three major *ras* genes has been determined. In addition, the sequence of the coding regions of K- and N-*ras* genes and of the entire H-*ras* gene is known. In 1983 the genomic organization and sequence of the H-*ras* gene was reported from three laboratories (Reddy, Capon et al., and Fasano et al.). The gene is contained within a 6.6kb BamHI restriction fragment and expresses a transcript of approximately 1kb containing four translated exons and untranslated flanking regions. The protein product has 189 amino acids and a molecular weight of 21kDa. McCoy and Weinberg (1986) found that K-*ras*

has a different organization, in that it occupies over 50kbp and has a total of five coding regions with two forms of exon 4, designated a and b. Because of alternate splicing, two different transcripts are present in the cell. However, over ninety percent of the transcripts contain only exon 4b. The remainder contain both 4a and 4b, but because a stop codon at the 5' end of 4b is recognized, only 4a is translated. Translation of exon 4b results in a 188 amino acid protein, whereas 189 amino acids are encoded by the transcript containing exon 4a. In either case, the protein has a molecular weight of 21kDa. McGrath et al. (1983) determined the sequences of the coding region of the K-*ras* proto-oncogene (K-*ras* 2) and of the entire K-*ras* pseudogene (K-*ras* 1). The N-*ras* gene from the neuroblastoma cell line SK-N-SH was cloned by Taparowsky et al. (1983). They identified four coding exons within approximately 15kbp of DNA, encoding a protein of 189 amino acids. Hall and Brown (1985) reported similar studies on cDNA clones derived from transcripts expressed by the human fibrosarcoma cell line HT1080. They found that HT1080 cells express two different transcripts, one 4.3kb in length, containing seven exons, and another 2kb in length, also containing seven exons. In both cases only four exons encode protein, resulting in a 189 amino acid product.

b.) Protein

Overall, the amino acid sequence identity among the three human *ras* gene products is greater than 90% (Valencia et al., 1991). The greatest divergence occurs in the carboxyl terminal domain encoded by exon 4. Furthermore, there is significant sequence similarity across widely divergent species. The implication of this level of sequence identity is that the amino terminal portion of the protein is responsible for some

function that is critical in normal cells. The p21 protein has been divided into several functional domains each defined by structural elements. Five regions in particular have been shown to be important for the binding of the guanine nucleotides and for interaction with the GTPase activating protein GAP (purified by Trahey and McCormick 1987), as well as downstream effector molecules (reviewed by Bourne et al., 1991). Determination of the structure of the ras proteins has resulted in a better understanding of their function and their role in malignant transformation. Wierenga and Hol (1983) compared the structure of wild-type H-ras p21 and the activated form encoded by the EJ and T24 H-*ras* genes. They showed that any amino acid other than glycine at position 12 results in changes in guanine nucleotide binding. In similar studies, Pai et al. (1989) determined the structure of H-ras p21 and deduced the changes induced by mutations at the codons known to activate oncogenic activity. In every case these mutations are predicted to affect the guanine nucleotide binding or the GTPase activity (both intrinsic and GAP-activated), lending support to the hypothesis that appropriate regulation of ras p21 is dependent on wild-type structure. Similarly, Krengel et al. (1990) showed that activating mutations impair normal guanine nucleotide binding and GTP hydrolysis. Further evidence of the importance of p21 structure came from studies by Alonso et al. (1990), who found that NIH-3T3 cells transformed by mutant *ras* have impaired coupling to phospholipase C in response to PDGF stimulus, whereas they show enhanced coupling in response to bradykinin.

Cellular localization is also important to the function of ras p21. In 1980, Willingham et al. showed by immunocytochemistry that the H-ras product is associated with the internal surface of the cell membrane.

This association occurs as a result of post-translational modification (reviewed by Grand and Owen, 1991), involving a carboxyl terminal tetrapeptide, CAAX (Cys, aliphatic amino acid, aliphatic amino acid, any amino acid). Subsequent work by Der and colleagues (Jackson et al., 1990) showed that post-translational modification is essential for transformation by K-*ras*.

3. Role of *ras* in Normal Cellular Function

ras gene expression is found in most cell types. In a study of ras p21 expression in human cells, Furth et al. (1987) detected the presence of p21 in almost all tissues. Furthermore, they found increased levels of expression in actively dividing cells, for example, squamous epithelium near the basal layer and colon crypt cells. Increased expression was also found in cell types with specialized membrane function, for example, neural cells and pancreatic secretory cells. Increased H-*ras* expression also occurs in liver cells after partial hepatectomy and during carcinogen-induced regeneration (Goyette et al., 1983). These data support the hypothesis that *ras* expression plays an important role in regulation of cell division.

It has been unclear whether there is any coordination of the expression of the various *ras* genes. Support for this notion comes from data by Nieto et al. (1987) in which H-ras p21 expression was shown to be increased in rat mammary gland during gestation whereas the level of K-*ras* expression remained constant. In contrast, Müller et al. (1983) found that although some proto-oncogenes are specifically expressed at different stages of mouse development, H- and K-*ras* are both stably expressed throughout embryonic development and post-natally.

The role of *ras* in normal growth and development remains unclear; however, in individual cells there is support that *ras* expression is required for cell division. Mulcahy et al. (1985) microinjected anti ras p21 antibody into cells that had been transformed by microinjection of large amounts of p21. The cells that received the antibody could not enter the S phase of the cell cycle, indicating that *ras* expression is required for cells to cycle.

B. Evidence for the Role of *ras* in Malignant Transformation

1. Evidence from Correlative Data

a.) Human tumor cells

The study of human tumor cells has provided strong evidence that *ras* activation plays a role in several tumor types. In some cases the importance of *ras* in malignant transformation can be inferred from the high incidence of *ras* activation in cells from a common origin. For example, Forrester et al. (1987) reported a 39% incidence of K-*ras* mutations in human colon tumor cells by the RNAse mismatch method, and Smit et al. (1988) found a 93% incidence of *ras* activation in pancreatic adenocarcinoma. On the other hand, in other tissues there is a weaker association between *ras* activation and malignancy. For example, Neri et al. (1988) reported N-*ras* activation in only 18% of a series of acute lymphoblastic leukemias and no *ras* activation in non-Hodgkin lymphoma. Although associative data implies, but does not prove, causality, one study provides stronger evidence that *ras* activation plays a causative role in transformation. Senn et al. (1988) found N-*ras* mutations in

malignant cells from five of eighteen patients with acute non-lymphocytic leukemia. These patients were treated, and some achieved remission. When the patients were re-assessed, the cells of the patients who had achieved remission had only wild-type N-*ras*. In contrast, the cells of one patient who did not achieve remission continued to show the same N-*ras* mutation that was detected prior to treatment. Taken together these data provide strong evidence for the role of *ras* activation in the genesis of certain tumors.

b.) Animal tumor cells

Experiments in carcinogen-treated animals also provide strong supporting evidence that *ras* activation can play a causative role in tumor development. One such study reported by You et al. (1989) compared K-*ras* mutations in spontaneous and chemically induced lung tumors from the strain A mouse. Mice of this strain have increased susceptibility to the formation of lung tumors. In cells from spontaneous and carcinogen-induced tumors, the incidence of *ras* activation was high (83-100%); however, there was a difference in the location of the activating mutation depending on whether the tumor was spontaneous or carcinogen-induced. Specifically, cells from spontaneous tumors showed a mix of mutations at codon 12 and codon 61 whereas cells from all methylnitrosourea-induced tumors showed mutations at codon 12, and 90% of cells from ethyl carbamate-induced tumors were mutated at codon 61. These data indicated the importance of *ras* activation in animal tumors, linked tumor induction by carcinogen treatment with K-*ras* activation, and added to existing data on H-*ras* mutation in DMBA-induced mouse mammary carcinogenesis (Dandekar et al., 1986) and polycyclic aromatic hydrocarbon-induced skin tumors in

mouse (Bizub et al., 1986).

Experiments using carcinogen-treated animal models have also provided some information regarding the timing of *ras* activation in the process of malignant transformation. In 1988 Pelling et al. studied *ras* mutations in mouse skin tumors that arose in animals treated only with a tumor promoter, in contrast to other studies in which animals had been exposed to DMBA, a mutagen, followed by treatment with a promoting agent. They found an H-*ras* mutation in more than 70% of benign and malignant tumors. Similar data were reported by Barbacid and colleagues (Kumar et al., 1990) in a mouse mammary tumor model. Mutations in H- and K-*ras* were detected as early as two weeks after initiating carcinogen treatment and several months before neoplastic cells were observed. A reasonable conclusion from these studies is that *ras* activation is an early change in malignant transformation and that cells containing this change can be targeted by other transforming agents such as tumor promoters.

c.) Cells in culture

Another line of correlative evidence for involvement of *ras* activation in malignant transformation has come from carcinogen treatment of both human and animal cells in culture. One such study by Miyamoto et al. (1990) showed that methyl nitrosourea-treated mouse mammary epithelial cells contained specific mutations in the K-*ras* gene. Furthermore, mutations could be detected in partially transformed, as well as in malignant cells, again supporting the notion that *ras* activation is an early event in the neoplastic process. Weissman et al. (1989) performed similar experiments in a mouse keratinocyte cell line. This cell line is epidermal growth factor(EGF)-dependent and can be induced to differentiate

terminally by the addition of calcium to the growth medium. These characteristics allowed the investigators to select EGF-independent, calcium-resistant transformants after treatment. In each case, the cells that were selected by their ability to differentiate following EGF stimulation contained *ras* mutations, implicating *ras* mutations in abnormal growth and differentiation.

2. Evidence from Insertion of Activated *ras* Genes

More direct evidence that *ras* plays a role in the malignant transformation comes from experiments in which transformation is brought about by insertion of activated *ras* genes. One example is from work reported by Bos and colleagues (Vousden et al., 1986), in which plasmids containing wild-type H-*ras* were treated with mutagens. The resulting activation of the *ras* gene was evidenced by focus formation in NIH-3T3 cells transfected with the treated plasmids. When the activated DNA was analyzed, point mutations were found at codon 12 and codon 61. The nature of the mutations was consistent with that typically found in DNA treated with the specific mutagenic agents used. This study serves as a bridge between correlative studies and transformation by the insertion of activated *ras* into cells. Other in vitro transformation studies strengthen support for the role of *ras* activation in multi-step transformation. For example, Harper et al. (1986) were able to complete the transformation of mouse papillomas by insertion of the activated H-*ras* gene isolated from the human EJ bladder carcinoma cell line. Similar studies on human fibroblasts in our laboratory have been discussed in earlier sections of this review (Hurlin et al., 1989). In this case, an immortal human cell line that expresses a transfected v-*myc* gene (Morgan

et al., 1991) was transformed to tumorigenicity after transfection of the T24 (EJ bladder carcinoma) H-*ras* oncogene, whereas a finite life span cell line, although morphologically transformed, eventually senesced, and did not form tumors (Hurlin et al.,1987). Others working with human fibroblasts have reported similar results using transfection with N-*ras* (Kinsella et al., 1990), suggesting that c-*myc*-induced immortality is a prerequisite to obtaining full transformation.

Evidence of the function of activated *ras* genes in bringing about the tumorigenic state was obtained by Kasid et al. (1985), who transfected a human breast cancer cell line with the viral H-*ras* gene. Prior to transfection, the cells, though fully transformed, were dependent on estrogen in order to form tumors. The *ras*-expressing transfectants no longer required estrogen to grow rapidly in culture, were not significantly inhibited by anti-estrogen compounds, and no longer required estrogen supplementation to form tumors in test animals. This suggests that the presence of activated *ras* bypasses the normal estrogen-regulated growth control pathways in breast cells.

3. Evidence from Reversion of *ras*-Induced Transformation

A third avenue by which the importance of *ras* activation to malignant transformation and the mechanisms involved have been determined is from observations of *ras*-transformed cells that have reverted to a less-transformed phenotype. Exemplifying this approach is a study by Schaefer et al. (1988) in which genomic DNA from human placenta was transfected into *ras*-transfected tumorigenic cells from rats. Some of the transfectants displayed partial reversion as evidenced by decreased growth in soft agar, morphological reversion, and decreased tumorigenic

potential. The reversion did not occur by a decrease in the expression of the activated *ras* gene. Although the investigators were able to identify the fragment of DNA that conferred the reverted phenotype, more specific identification was not reported. A repressor of K-*ras*-induced transformation was identified through a series of experiments involving NIH-3T3 cells transformed by v-K-*ras*. Noda et al. (1989) found that reversion to a non-transformed state was mediated by plasmid DNA introduced into the cells. The gene responsible (termed *Krev*-1) was shown by Kitayama et al. (1989) to encode a 184 amino acid protein with 50% sequence identity to K-*ras*, as well as similar guanine nucleotide binding and GTPase activity. By performing site specific mutagenesis studies, Zhang et al. (1990) localized the sequences required for reversion to the effector domain (residues 32-40), leading to the speculation that *Krev*-1 acts by competing with K-*ras* for downstream targets. Another example of reversion was reported by Shindo-Okada et al. (1989). Their studies focused on identifying chemotherapeutic agents by their ability to cause reversion of transformation in H-*ras*-transformed NIH-3T3 cells. One compound, the antibiotic azatyrosine, caused a high frequency of reversion in both *ras*- and *raf*- transformed NIH-3T3 cells without causing significant growth inhibition of normal NIH-3T3 cells. The antibiotic did not interfere with the expression of the transfected activated *ras* gene. Furthermore, if the agent was removed, reversion was stable for at least three months. The mechanism by which this antibiotic acts was not determined. Because of the high frequency of reversion the authors concluded that genetic mutation was unlikely. Since reversion was attained in *ras*- and *raf*-transformed cells one can speculate that azatyrosine acts within the signal transduction pathway that involves the

normal counterparts of the *ras* and *raf* genes.

C. Mechanism of *ras*-Mediated Transformation

1. Point Mutation-Induced Changes in *ras* p21 Activity

Insights into the mechanisms by which activated *ras* genes function in the process of malignant transformation can be gained by investigating the biochemical activities of *ras* gene products in their wild-type form, their activated form, or those that have been altered in such a way that specific functions are lost. The conserved functions of these proteins are guanine nucleotide binding, GTPase activity, effector interaction, and membrane localization. Therefore, it is reasonable to hypothesize that an alteration in DNA that causes the protein to lose one or more of these functions would lead to activation or inactivation of the ability of *ras* to cause transformation. Clanton et al. (1986) investigated the guanine nucleotide binding capacity and transforming activity of *ras* gene products. They created several vectors, each containing a specific mutation within the v-H-*ras* gene that was predicted to alter guanine nucleotide binding. Only two of the mutants, one that substituted lysine for asparagine at codon 116, and another that substituted tyrosine for asparagine at codon 116, showed loss of guanine nucleotide binding. These two mutants were also found to be deficient in autokinase activity (a property of the activated viral *ras* oncogenes), and were unable to transform NIH-3T3 cells to focus formation. The investigators concluded that guanine nucleotide binding is required for transforming function of *ras*-encoded products. In contrast, Der et al. (1986), created H-*ras* mutants at codons 61, 116, 117, or 119 that showed decreased guanine

nucleotide binding, but no change in transforming capacity. However, the mutants they tested contained different amino acid substitutions than were in Clanton's study, and were not deficient in autokinase activity. It is reasonable to conclude from these two studies that specific amino acid changes interfere differently with protein function, and that absolute loss of guanine nucleotide binding is required in order to lose transforming activity.

It is established that ras p21 cycles between its GTP-bound ("on state") and GDP-bound ("off state"), and that the steady state ratio of GTP- to GDP-bound p21 is greater in actively dividing cells than in quiescent cells (Haubruck and McCormick, 1991). Mutations in *ras* that result in decreased p21 GTPase activity (and therefore increased GTP-bound p21) might be expected to cause transformation. The earliest reported example of an association between GTPase activity and transformation is by Sweet et al. (1984), who found that the product of the T24 *ras* gene has only one-tenth the GTPase activity of wild-type ras p21. Manne et al. (1985) compared the GTPase activity of wild-type and activated ras p21 mutants using a highly sensitive assay that employed bacterially expressed, purified p21. All of the activated products showed decreased GTPase activity. However, data by Der et al. (1986) showed that although alteration in normal GTPase activity may be necessary for transformation, it is not sufficient, since they could not establish a direct correlation between GTPase activity and transforming capacity.

Clearer understanding of the mechanism by which GTPase activity is decreased in activated ras p21 came from work by McCormick and colleagues (Adari et al., 1988) who showed that the GAP protein (identified by Trahey and McCormick in 1987) interacts with p21 at its effector binding domain,

and that *ras* genes containing activating mutations encode products that are resistant to the GAP-mediated increase in GTPase activity. However, Srivastava et al. (1989) showed that certain deletion mutants of *ras* that abolish interaction with the antibody Y13-259, also show decreased interaction with GAP and have diminished transforming capacity. This result runs counter to expectations if GAP is the only protein which interacts with the ras p21 effector binding domain, since one would expect loss of GAP interaction to lead to a decrease in GTPase activity and augment transforming capacity. Evidence of other effector interactions comes from data showing increased inositol phospholipid breakdown in cells containing activated *ras* (Hancock et al., 1988). Recent data have also shown that ras p21 interacts with other proteins at the effector binding domain. The identity of these so-called "downstream" molecules and their role in transformation have only recently been reported and will be discussed in the appendix section of this dissertation.

2. Cooperation between *ras* and Other Oncogenes

Because malignant transformation is a process requiring many steps, it is not surprising that multiple genetic alterations are required, and that these changes are cooperative. This hypothesis has been supported by several experiments. For example, Reed et al. (1990) co-transfected the oncogenes *bcl2* (thought to encode a protein with GTP-binding activity) and H-*ras* into rat embryo fibroblasts. The transfectants were transformed as evidenced by focus formation, growth in soft agar and tumorigenicity. They also performed a series of co-transfections, including *ras* and *E1A*, *ras* and c-*myc*, *bcl*-2 and *E1A*, and *bcl*-2 and c-*myc*. Co-transfectants expressing *ras* were malignantly transformed, whereas those expressing *bcl*-

2 were not, suggesting that although *ras* and *bcl-2* have similar biochemical properties, they act in distinct cellular regulatory pathways. The importance of *myc* and *ras* cooperation was shown by Sklar et al. in 1991. In these studies *ras*-transformed tumorigenic NIH-3T3 cells transfected with a vector containing anti-sense c-*myc* were found to revert to a less tumorigenic, less transformed phenotype. Transfection of anti-sense *myb* and *jun* did not produce reversion, suggesting that *ras* transformation occurs through a *myc*-mediated pathway. However, Schütte et al. (1989) showed that c-H-*ras* and c-*jun* can cooperate to transform primary rat embryo cells. Binétruy et al. (1991) showed that expression of an activated H-*ras* caused increased transcriptional activity of the c-jun protein, associated with its hyperphosphorylation. They concluded that expression of activated *ras* initiates a protein kinase cascade resulting in a hyperphosphorylated, more active c-jun protein. Further studies are needed to form meaningful conclusions about the role of these oncogenes and the means by which their products cooperate to produce transformation.

3. Alterations in *ras* Expression

There is evidence that the transforming capacity of the *ras* oncogenes can be altered by increased expression as well as by point mutation as is true of several other oncogenes. Duesberg and colleagues (Chakraborty et al., 1991) showed that even a wild-type *ras* gene is capable of transforming the mouse fibroblast line C3H/10T1/2, although the presence of activating mutations greatly enhances transforming capacity. It should be noted; however, that this cell line has an infinite life span and is transformed with a frequency as high as 3% after carcinogen

treatment. Therefore, it is likely that other genetic changes in that cell line are cooperating with increased *ras* expression to cause transformation. Similar experiments performed by Ricketts and Levinson (1988) showed that over-expression of the wild-type *ras* gene can partially transform Rat-1 cells, but only when the level of expression are very high. Several additional studies have shown that transfection of activated *ras* genes, either under the control of an inducible promoter (Seyama et al. 1988), or under selective conditions leading to gene amplification (Sistonen et al. 1987), transforms recipient cells to a greater or lesser extent, depending on the level of expression achieved. Investigators have also examined the effect of alterations in the level of expression of both wild-type and mutant endogenous *ras*. For example, Cohen and Levinson (1988) studied the T24 H-*ras* gene and showed that in addition to the mutation at codon 12 known to result in activation, there is an additional mutation in the last intron. This latter mutation results in a ten-fold increase in expression over that ordinarily found in cells. Without that mutation, the T24 gene shows decreased transforming efficiency. Comparison of this region of the T24 H-*ras* gene to that of H-*ras* genes from a panel of human genomic DNA showed that this alteration is not typically found in H-*ras* genes, leading to the speculation that the intron sequence alteration is a second somatic event that is important for full transforming capacity. In subsequent experiments to determine the role of the altered sequence, Cohen et al. (1989) found that the mutation abolishes a splice site, and that this results in an altered transcript encoding a truncated 19kDa protein. This altered transcript also exists in cells with wild-type H-*ras* gene, but is present only in low levels. Since the truncated protein lacks the normal carboxyl terminus required

for normal activity, it is thought to be non-functional and to suppress the expression of the normal protein. Loss of this splice site is expected to result in an increase in p21 expression. In the case of T24, this over-expression should result in transformation. Hashimoto-Gotoh et al. (1988) have reported other regulatory elements in the first intron of H-*ras* that appear to be conserved regions found in several proto-oncogenes, leading to the speculation that the expression of these proto-oncogenes is tightly regulated, and that derangements in this regulation contributes to transformation.

Coordination of expression of the different *ras* genes is also thought to play a role in *ras*-mediated transformation. Bizub et al. (1987) studied the expression of H- and K-*ras* in epithelial cells at several different stages of transformation and found an abundance of expression of H-*ras*. In view of many other reports of the frequency of H-*ras* activation in chemically-induced mouse skin tumors, these investigators hypothesized that cells in which one of the *ras* genes is preferentially expressed are a target for transformation by agents that can mutate that particular *ras* gene. In support of this idea, they also cite unpublished data on the increased expression of N-*ras*, and the increased frequency of mutations in that gene, that are found in hematopoietic malignancies. In contrast, Campisi et al. (1984) showed that although K-*ras* was mutated in a mouse fibroblast cell line transformed by chemical carcinogens, cell cycle regulated expression of K-*ras* was not lost, whereas normal regulation of *myc* expression was altered in the transformed cells. Clearly more work needs to be done to gain a clearer understanding of the normal control of *ras* gene expression and the role of altered regulation of expression in malignant transformation.

V. Methods for Determining the Presence of Activated *ras* Genes

A. Early Methods

Early methods for detecting cellular changes associated with malignant transformation were based on the rationale that malignant transformation had a genetic basis, and that certain in vitro phenotypic changes correlated with transformation. However, the specific genetic changes responsible for transformation had not been identified. Therefore one approach was to transfer DNA from a transformed cell line into test cells, observe them for the known transformation-associated phenotypes, isolate the DNA from the cells that had acquired this phenotype, and search for the specific oncogene that was responsible. Weinberg and colleagues (Shih et al., 1979) reported such an experiment, using DNA from rat cells transformed by carcinogen treatment. The DNA was transferred into a mouse cell line, NIH-3T3, known to take up large quantities of DNA and to exhibit specific transformation-associated changes in its normal growth. Some of the recipient cells were, indeed, transformed, forming dense piled-up areas of cell overgrowth termed foci. This assay was subsequently used to identify the presence of transforming genetic material. As methods developed to identify specific regions of DNA (for example Southern analysis and probing with known fragments of DNA) it became clear that *ras* genes bearing specific mutations were capable of transforming NIH-3T3 to focus formation. The NIH-3T3 focus assay, and variants utilizing transformation to tumorigenicity (Blair et al., 1982) were used to identify specific activating *ras* mutations in a number of tumor cells derived from various human tissues. These mutations are located at codons 12, 13, 59, 60, 61, 117, and 119. This method has some

limitations that have decreased its utility for detecting mutations in *ras* genes. First, its basis is the ability of a *ras* gene carrying a mutation to cause a recognizable phenotypic change, i.e.. focus formation. Therefore, any mutation that is capable of activating the *ras* gene, but that does not result in focus formation would be missed. This was documented by Higashi et al. (1990), who showed that a mutation at codon 146 in the K-*ras* gene resulted in no significant increase in focus formation in NIH-3T3 cells, but did efficiently transform a hamster cell line. Secondly, this method is a screening tool, and even when focus formation is induced, the DNA fragment responsible must be identified by other methods. Finally the method is cumbersome and time-consuming, and as more sophisticated approaches have evolved, this method has been used less frequently.

B. Later Methods

The invention of the polymerase chain reaction (PCR) method of amplifying specific segments of DNA, which is capable of synthesizing large quantities of DNA from a very small starting sample (often as little as one cell), has resulted in a number of new techniques to search for the presence of mutations in *ras* genes. Among the most commonly used is mismatch oligonucleotide hybridization. In this method, a fragment of DNA containing a sequence of interest is specifically amplified, and probed with one of several radiolabeled oligonucleotides designed to match either the wild-type sequence, or one of the possible mutants. By utilizing highly stringent hybridization conditions, the sequence of the amplified fragment can be inferred from the oligonucleotide with which it can still hybridize. Mutations in *ras* genes of many human tumor cells

have been identified by this method. In one example, Vogelstein and colleagues (Sidransky et al., 1992) were able to identify K-*ras* mutations in sloughed cells obtained from stool of patients with colon cancer. These mutations corresponded to the K-*ras* mutations observed in the cells taken from colon adenocarcinoma removed from those patients.

Sequence analysis can also be performed on amplified DNA. Sequence determination has the additional benefit of identifying wild-type and mutant fragments simultaneously. In one example, Mane et al. (1990) determined the sequence of an N-*ras* fragment from patients with acute myelogenous leukemia. They showed that a mutation in the amplified DNA could be detected by sequencing even when the mutant DNA had been diluted ten-fold with the corresponding wild-type DNA.

Some PCR-related methods combine the amplification with analytical methods. These include the use of amplification primers that are designed to anneal specifically to DNA containing a mutation (Ehlen and Dubeau, 1989), as well as primers designed to form a specific restriction endonuclease recognition site when annealing to target DNA containing a specific mutation (Kumar and Barbacid, 1988).

Mutations of *ras* genes can also be detected at the level of RNA or protein expression. These methods rely on specificity of association of an RNA probe for wild-type or mutated *ras* messenger RNA, or on the specific interaction between an anti-p21 antibody and wild-type or mutant protein. An example of RNA detection was reported by Lang et al. in 1989 who used radiolabeled probes to detect H-*ras* message in situ, avoiding the additional step of extraction of RNA from the cells of interest. One advantage of such a method is that it allows the screening of large numbers of cells for a specific mutation. Also, detection at the mRNA

level indicates that mutant genes are being expressed and, therefore, are likely to have biological function.

An example of detection of activated ras p21 utilizing monoclonal antibodies to detect mutant p21 was reported by La Vecchio et al. (1990). These investigators generated two monoclonal antibodies to a synthetic peptide corresponding to residues 5-16 of ras p21 that contained a specific alteration (substitution of aspartate) at codon 13. One of these monoclonal antibodies specifically immunoprecipitated cellular ras p21 containing this altered amino acid. This led to the conclusion that this monoclonal antibody would be useful for rapid detection of ras p21 activated by the presence of aspartate at residue 13. Investigators have also reported that some mutant ras proteins are detectable by alteration of electrophoretic mobility of ras protein immunoprecipitates. However, Andeol et al. (1988) showed that some cell lines known to contain mutant activated *ras* did not express an abnormal protein detectable by an altered immunoprecipitation pattern. Therefore, this method cannot be expected to find all activating mutations.

All of the above methods for the detection of activated *ras* rely on previous data associating mutation at specific codons with activation of the oncogene. Therefore, mutations which have not been identified by the early functional assays (for example focus formation), are not routinely searched for, and may be missed by these specific methods. Until recently, only sequence determination of the entire coding region could provide data that were not limited by this historical bias. A very new screening method is capable of detecting the presence of a single point mutation in an amplified fragment and can conveniently be used to screen the entire coding region. This method, single strand conformation

polymorphism (SSCP), was shown by Suzuki et al. (1990) to be sensitive in detecting single nucleotide alterations in one strand of an amplified fragment in a series of *ras* genes from a number of cell lines known to contain activating point mutations. This method does not specify the nature or the precise location of the mutation, but does provide promise as a screening method to search large numbers of cells for mutations throughout the coding region. Any cells found to contain a mutation by this method can then be analyzed further using sequencing of the relevant fragment to determine the nature and exact location of the mutation.

In general, several issues need to be considered when determining the most appropriate method by which *ras* gene mutations are detected. One question is the type of material to be tested. For example, if the samples are to come from archival tissue, a method would need to be based on detection of DNA rather than RNA or protein since it is much more stable. Also, a method would need to be able to differentiate between malignant and normal cells in the sample. If the sample consists of a homogeneous cell line, the question of separating normal and transformed tissues need not be considered. Another issue is whether the study is intended to screen samples for possible mutations, or whether the effect of specific alterations on function is being investigated. In the former case, a more general method for detecting mutations (such as sequence determination of the coding region) might be expected to be most sensitive. In the latter case, methods designed to detect mutations in specific codons would be sufficient. In the study that I carried out for this dissertation, the purpose was to determine the role of *ras* activation in a specific subset of cells. Therefore, the determination of the sequence of the entire coding region was considered to provide the most

sensitive analysis. It was also judged to be feasible because the number of cell lines to be analyzed was relatively small.

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CHAPTER II

Sequencing PCR-amplified *ras* Genes to Determine their Importance to the Malignant Transformation of Human Fibroblasts

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ABSTRACT

Activation of *ras* proto-oncogenes by mutation at specific codons has been documented in a variety of human malignancies. To determine the extent to which *ras* activation plays a role in the malignant transformation of human fibroblasts, we sequenced the coding region of the H-, K-, and N-*ras* genes from a series of human fibroblast cell lines, including normal diploid human fibroblasts, two morphologically normal non-tumorigenic infinite life span cell strains originated in this laboratory, spontaneous and carcinogen-induced malignant transformants of the latter, and cell lines derived from human fibrosarcomas. Direct sequencing of the PCR product was carried out using the dideoxy chain termination method. A total of twelve cell lines or strains were analyzed for *ras* mutations. Of the eight that are malignant, two fibrosarcoma-derived cell lines contained an activating *ras* mutation. One mutant cell line, 8387, has a G to C transversion that causes an Ala to Pro amino acid substitution at codon 146 in one allele of the Ki-*ras* gene. This same mutation has been reported to activate Ki-*ras* in Hut-14 cells, an 8387 derivative. The other mutant cell line, HT1080, has a C to A transversion that causes a Gln to Lys amino acid substitution at codon 61 in one allele of the N-*ras* gene that has been reported previously. The remaining cell lines or strains contain no mutations in *ras*. The mutation frequency in the fibroblastic cells tested in this study is consistent with one other report on *ras* activation in human sarcomas in the literature. Our results

show that although *ras* oncogenes can play a role in the development of fibrosarcomas in humans, some other changes must be able to substitute for *ras* activation in the malignant transformation of human fibroblasts. Also, methods of detecting *ras* activation that concentrate on the "classic" activating codons may miss activated genes. Further studies are planned to test for alterations elsewhere in the *ras* p21 signalling pathway using cells that contain only wild-type *ras* genes.

INTRODUCTION

It is well established that malignant transformation of human cells occurs by a multi-step process in which an individual cell accumulates a number of stable heritable changes that together result in the acquisition of characteristics such as immortality, autonomous growth, and metastatic capacity. The steps in this process have been linked to alterations in a number of genes. Among these, the *ras* family of proto-oncogenes, the first of which was discovered as the cellular homolog of the transforming gene of the Harvey sarcoma virus [1], has been widely studied. The three major *ras* genes, Ha-, Ki-, and N-*ras*, encode 21kDa proteins that associate with the internal surface of the cell membrane, bind guanine nucleotides, and have an intrinsic GTPase activity. Studies have shown that ras p21 cycles between its GTP-bound ("on" state) and GDP-bound ("off" state) forms (reviewed by [2]); however, the steady-state ratio of GTP- and GDP-bound p21 is greater in actively dividing cells than in quiescent cells. This observation, along with data showing the requirement for *ras* expression in serum-stimulated cell division [3], supports the hypothesis that wild-type *ras* p21 is involved in transduction of mitogenic signals from protein growth factors (reviewed by [4]).

When mutated at specific codons (most commonly at 12, 13, 59, 60, 61, and 117 [5]) *ras* proto-oncogenes become activated oncogenes. Several lines of evidence point to a role for activated *ras* oncogenes in malignant transformation. First, mutated *ras* genes have been shown to be present in

a number of malignant human cells [6]. Second, *ras* activation has been associated with induction of malignant transformation of animal cells in culture by chemical carcinogens [7] and radiation [8]. Third, the introduction of activated *ras* genes into cells in culture has been shown to induce transformation in animal [9] and human [10] model systems. Finally, loss of expression of activated *ras* genes has been shown to result in reversion to a less-transformed phenotype [11-13].

Cultured human and animal fibroblasts are commonly used as a model system in which to study the process of malignant transformation, primarily because the relative ease of propagation has allowed their growth and phenotypic properties to be well defined. In this and other laboratories, *ras* oncogenes have been established as transforming agents when transfected into normal diploid human fibroblasts [14] and immortal human fibroblasts [15-17]. However, there is a paucity of data on the role of endogenous *ras* genes in malignant transformation of human fibroblasts. Studies have implicated *ras* activation in some human mesenchymal cell malignancy (e.g. myeloid disorders [6]), but in one study on a series of sarcomas, no *ras* mutations were found [6]. With the development of more sophisticated methods for detection of *ras* activation, data on the incidence of *ras* activation in many cell types has changed; either showing an increased incidence, as in ovarian carcinoma [18, 19] or a decreased incidence, as in bladder cancer [20]. It is reasonable to hypothesize that the incidence of *ras* activation in sarcoma cells is also different than reported previously.

To gain a clearer understanding of the role of *ras* genes in the malignant transformation in human fibroblasts, we have searched for *ras* activation in two series of human fibroblast-derived cell lines. The

first series is comprised of established cell lines from human fibrosarcomas (Table 1). By working with a pure population of cultured cells one avoids the of problem separating normal and malignant cells. Although one might be concerned about the possibility that long-term propagation in culture has resulted in selection artifacts, these cell lines have remained relatively stable in terms of chromosome content and phenotype. Furthermore, as all but one continue to be tumorigenic, they most likely contain the changes that originally resulted in their transformation.

Table 1. Cell lines derived from human fibrosarcomas

Cell line	Origin	Original Reference
SL84	neonatal foreskin*	[21]
HT1080	fibrosarcoma†	[22]
SW982	fibrosarcoma‡	[23]
8387	fibrosarcoma	[23]
NCI	fibrosarcoma	[23]
VIP:FT	spontaneous malignant transformant	[24]

Table 1. Fibrosarcoma-derived cell lines used for determination of *ras* mutation. *Normal cell line used as a negative control. †Known to carry an activating N-*ras* mutation [25]. ‡ Originally derived from a human fibrosarcoma, but no longer tumorigenic.

The second series (listed in Table 2) is comprised of a number of cell strains derived from an immortal cell strain generated in this laboratory [26]. This series includes the immortal near-diploid non-malignant parent human fibroblast strain, and several malignant transformants derived from that immortal strain that have occurred either spontaneously or as a result of treatment with chemical carcinogens. The origin and characteristics of the individual strains used in this study are outlined in Figure 1. The specific cell strains from the lineage that were studied were chosen because they possess characteristics in culture that are similar to those of cells transformed by transfection of activated *ras*, because of evidence from immunoprecipitation of p21 that their *ras* genes are altered (McCormick, unpublished observation), or because they exhibit changes in growth factor requirements that are hypothesized to result from *ras* activation. Since this series forms a lineage, it could also allow us to determine whether *ras* activation is an early or late event in malignant transformation.

We examined these various cell strains for *ras* activation by determining whether or not mutations were present in the coding region of the Ha-, Ki-, and N-*ras* genes. Direct sequencing of PCR-amplified cDNA was performed, since sequencing provides information on the location and kinds of mutations present in a cell, and also indicates whether one or both alleles are altered. We considered it important to determine the sequence of the entire coding region, particularly in view of recent developments in describing the complexity of *ras*-mediated

Table 2. Cell strains from the MSU-1 lineage

Name	Treatment	Selection	Tumor Morphology	Unusual Features
MSU-1.1	v-*myc*	life span	NA*	-2 marker chromosomes† -slight growth advantage
MSU-1.2	low Ca^{2+}	clonal growth	NA	novel phospho-protein (PDGFR‡?)
CSV0.20/ ST1	BPDE§	soft agar	spindle cell	
L55I-3T	MNNG¶	-focus formation -soft agar	round cell	altered p21 immuno-precipitation pattern
SD532AC 4MT/T2	ENU⊥	morphology	round cell	altered p21 immuno-precipitation pattern
DY6:16. 3C10C/T2	BPDE	focus formation	giant cell	

Table 2. Cell strains from the MSU-1 lineage used for determination of *ras* mutations. * NA, non-applicable since this strain does not form tumors. † This parental strain in the series has an infinite life span in culture, contains two marker chromosomes, and grows slowly in serum-free medium, but in all other respects it is similar to normal human fibroblasts. ‡ PDGFR, platelet-derived growth factor receptor. § BPDE, (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (a reactive metabolite of benzo[*a*]pyrene). ¶ MNNG, *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine. ⊥ ENU, ethylnitrosourea.

Figure 1. MSU-1 Lineage

Members of the MSU-1 lineage of cell strains used in this study. In addition to the cell strains shown here, there are several spontaneous, oncogene-transformed and carcinogen-induced malignant transformants of MSU-1.1. See text and Table 2 for more complete descriptions and references pertaining to the specific strains used in this study.

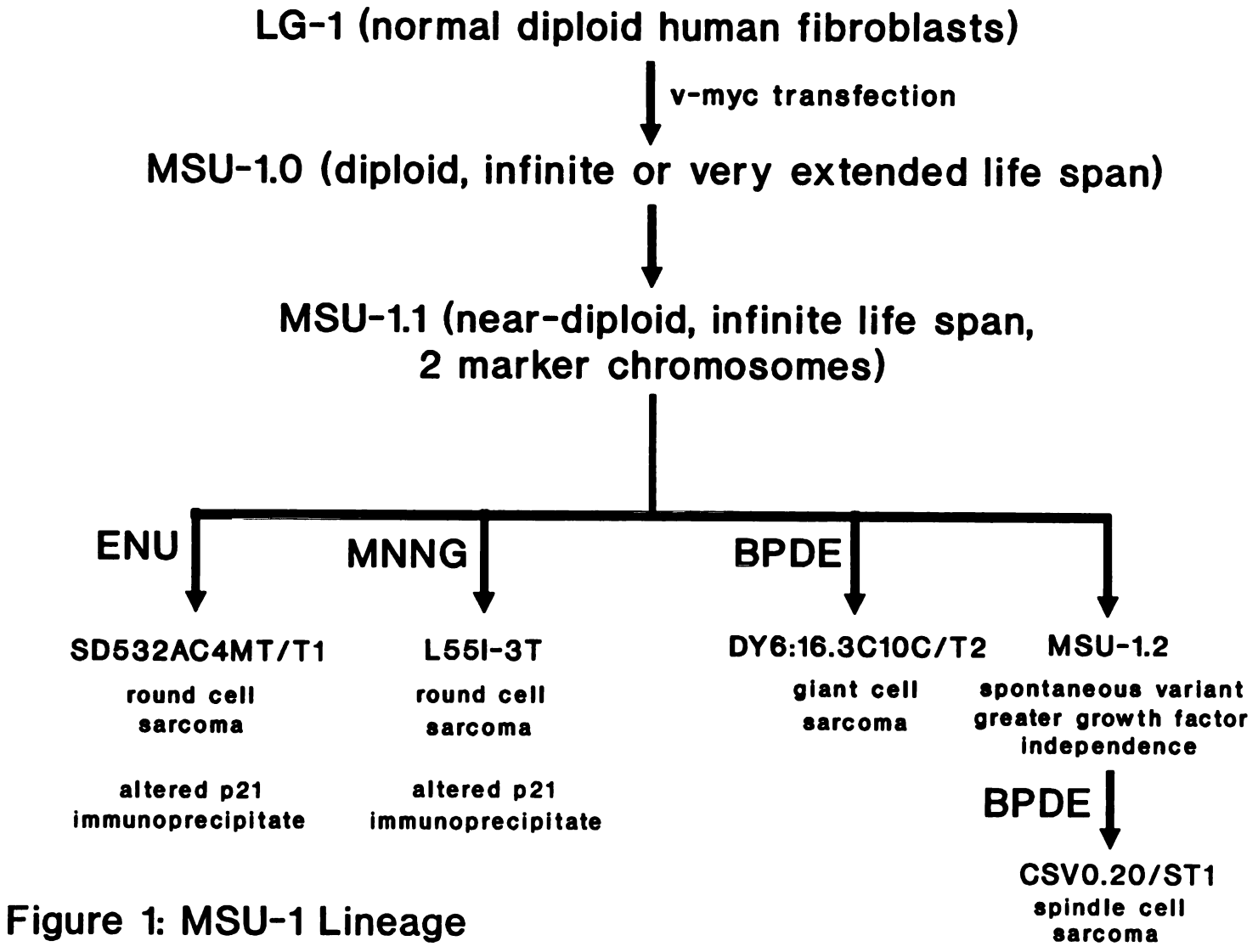

Figure 1: MSU-1 Lineage

signalling pathways [27]. The so-called classic activating codons have been determined by their ability to transform NIH-3T3 cells to focus formation. It is possible that mutations at other locations, for instance in the effector domain, are also important, but these would not be detected by methods concentrating only on the codons that have previously been reported to cause activation. Our results indicate that *ras* activation is not required for the malignant transformation of human fibroblasts. Further studies are needed to determine what changes substitute for *ras* activation in these cells.

MATERIALS AND METHODS

Routine Cell Culture

Cells were grown routinely in 5% CO_2 humidified air at 37°C on plastic in Eagle's minimal essential medium (Gibco, Gaithersburg, MD) supplemented with 0.2 mM aspartic acid, 0.2 mM serine, 1.0 mM Na pyruvate, and with 10% supplemented calf serum (HyClone, Logan, UT), penicillin (100 U/ml)-streptomycin (100 ug/ml), and hydrocortisone (10 ug/ml).

RNA Extraction

Total RNA was extracted by the acid guanidinium thiocyanate-phenol-chloroform method [28]. Briefly, cells in log-phase growth were rinsed with ice-cold PBS. The denaturing solution was added (4 M guanidinium thiocyanate, 25 mM Na citrate pH 7.0, 0.5% Na lauryl sarcosine, and 0.1 M 2-mercaptoethanol) and the cell lysate was scraped into a centrifuge tube. To the lysate, 2 M Na acetate pH 4.0, water-saturated phenol, and chloroform:isoamyl alcohol (24:1) were added in succession, with mixing at

each step. The suspension was centrifuged for 10 min. at 5,000 rpm. The aqueous top layer containing the extracted RNA was removed and precipitated with an equal volume of isopropanol at -20°C for at least one hour (typically overnight). The RNA was centrifuged for 20 min. at 5,000 rpm to form a pellet, washed with 75% ethanol and resuspended in water. The amount of RNA was determined from the OD_{260nm}. RNA was distributed into 80-100 ug aliquots, vacuum-dried and stored at -20°C until use.

Preparation of first strand cDNA

Total RNA (20 ug) was dissolved in 5 ul cDNA cocktail (50 mM Tris-HCl pH 8.3, 75 mM KCl, 10 mM DTT, 3 mM $MgCl_2$, 0.5 mM each of dATP, dCTP, dGTP, dTTP (Boehringer Mannheim Biochemicals, Indianapolis, IN), 100 ng oligo dT primer (Pharmacia, Piscataway, NJ), 15 U RNAse inhibitor (Promega, Madison, WI), and 200 U M-MLV reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD)) and incubated at 37°C for 1 hr. The samples were either amplified immediately or stored at -20°C for up to one week.

Second strand cDNA synthesis and in vitro amplification using the Polymerase Chain Reaction (PCR)

PCR amplification of the cDNA was carried out using a modification of the method of Saiki et al. [29]. 45 ul of PCR cocktail (10 mM Tris-HCl pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.001% gelatin, 0.4 mM each of dATP, dGTP, dCTP, dTTP (Boehringer Mannheim Biochemicals, Indianapolis, IN), 50 ng each of 5' and 3' PCR primers specific to the flanking sequences of the coding region of Ha-, Ki-, or N-*ras* (Fig. 2), and 2.5 U Taq polymerase (Perkin-Elmer Cetus Corp., Norwalk, CT)) was added to 5 ul of single-

stranded cDNA preparation. Cycling was performed in a Perkin-Elmer Cetus (Norwalk, CT) thermal cycler as follows: initial denaturation 94°C for 5 min. followed by 30 cycles amplification (94°C 1 min., 60°C 1 min., 72°C 2 min.) and finished with 72°C for 7 min. to complete extension. After the first 30 cycles, unincorporated nucleotides and primers were removed using Centricon-30 microconcentrators (Amicon, Danvers, MA). 10% of the concentrated sample (5 ul) was amplified a second 30 cycles under the same conditions using a new set of primers internal to those used for the first 30 cycles. After the second round of 30 cycles, the unused primers and nucleotides were again removed in a Centricon 30 microconcentrator (Amicon, Danvers, MA) and stored at -20°C prior to direct sequencing. Amplification was verified by agarose mini-gel and ethidium bromide staining.

Direct sequencing of PCR-amplified cDNA

Sequencing of amplified cDNA was carried out using a modification of the dideoxy chain termination method [33]. 100 ng sequencing primers (Fig. 2) were end-labeled with 20 U T4 polynucleotide kinase (New England Biolabs, Beverly, MA) and 100 uCi γ-^{32}P-dATP (Dupont New England Nuclear, Boston, MA) according to United States Biochemical Corporation (USB, Cleveland, OH) protocols for Sequenase version 2.0. Approximately 100 ng of amplified double-stranded DNA was annealed to 2 pmol end-labeled primer and reactions carried out with 3.25 U Sequenase version 2.0. Samples were separated on 8% polyacrylamide, 7 M urea gels. Gels were exposed to autoradiograph film (Kodak, Rochester, NY) for 12-36 hours. Sequences were compared to published sequences of Ha-, Ki-, or N-*ras*. Mutations were verified by repeating sequence determination with a different primer.

Figure 2: Oligonucleotide primers used for PCR and sequencing.

For concentrations used see Materials and Methods. Numbers in parentheses refer to the location of that primer in the gene or transcript: for Ha-*ras* [30]; for Ki-*ras* [31]; for N-*ras* [32]. Primer KSEQ2 spans the exon 1-exon 2 splice site. Underlined bases indicate where the sequence of the primer differs from the published sequence. These changes were made to have a BamHI recognition site.

Gene	PCR Primers	Sequencing Primers
Ha-*ras*:		
	HPR1 (forward)	HSEQ1 (forward)
	5′-GCCCGAATTCGCAGGTGGGGCAGGA-3′	5′-TGCCTGTTGGACA-3′
	(1603) (1627)	(2087) (2099)
	HPR2 (reverse)	HSEQ2 (forward)
	5′-AAGGGATCCTGCTGACCGCAGGCCA-3′	5′-GAACAAGTGTGAC-3′
	(3457) (3433)	(2435) (2447)
	HPR3 (forward, nested)	HSEQ3 (reverse)
	5′-GGAGGACCCCGGGCCGCAGG-3′	5′-CTCAAAAGACTTG-3′
	(1637) (1656)	(2209) (2197)
	HPR4 (reverse, nested)	
	5′-CCGGCAGGGGCGGGGAGCCG-3′	
	(3427) (3408)	
Ki-*ras*:		
	KPR1 (forward)	KSEQ1 (reverse)
	5′-GTACTGGTGGAGTATTTGAT-3′	5′-TCGAGAATATCCA-3′
	(6282) (6301)	(19528) (19516)
	KPR2 (reverse)	KSEQ2 (forward)
	5′-ACAGGCATTGCTAGTTCAAA-3′	5′-TAGAG/GATTCCTA-3′
	(37501) (37482)	(6483) (19476)
	KPR3 (forward, nested)	KSEQ3 (forward)
	5′-AGTGTATTAACCTTATGTGT-3′	5′-AGAGTTAAGGACT-3′
	(6302) (6321)	(21352) (21364)
	KPR4 (reverse, nested)	
	5′-CTGGGAATACTGGCACTTCG-3′	
	(21352) (21364)	
N-*ras*:		
	NPR5 (forward)	NSEQ1 (reverse)
	5′-ACTCGTGGTTCGGAGGCCCA-3′	5′-CCTGTCCTCATGT-3′
	(-205) (-186)	(223) (211)
	NPR6 (reverse)	NSEQ2 (forward)
	5′-TTGTACTAAACTACTGAGAG-3′	5′-TGGACATACTGGA-3′
	(724) (705)	(257) (269)
	NPR1 (forward, nested)	NSEQ3 (forward)
	5′-AGGCGCCTGGCAGCCGACTG-3′	5′-AAGTGTGATTTGC-3′
	(-165) (-146)	(349) (361)
	NPR2 (reverse, nested)	NSEQ4 (reverse)
	5′-AGCAGGAGCTTCTCTGTGAG-3′	5′-ATACACAGAGGAA-3′
	(694) (685)	(243) (231)
	NPR3 (forward, nested)	
	5′-CCGGTCTGTGGTCCTAAATC-3′	
	(-65) (-46)	
	NPR4 (reverse, nested)	
	5′-TACTTCTCCTCCAGGAAGTC-3′	
	(654) (635)	
	NPR7 (forward, nested)	
	5′-TGTCCAAAGCAGAGGCAGTG-3′	
	(-45) (-26)	
	NPR8 (reverse, nested)	
	5′-AGGACCAGGGTGTCAGTGCA-3′	
	(634) (615)	

Figure 2.

Cycle sequencing:

The 5' 200 base pairs of the N-*ras* cDNA was sequenced using the "fmol" sequencing kit (Promega, Madison, WI) according to the manufacturers instructions and with ^{32}P end-labeled primers (Dupont NEN, Boston, MA). Specific cycling conditions varied with the length and GC content of the various primers.

RESULTS

Determination of the status of the *ras* genes in cells from a series of fibrosarcoma cell lines:

We sequenced the entire coding region of the Ha-, Ki-, and N-*ras* genes of these cell lines. Two mutations were seen in this series of cell lines. The first, a mutation in N-*ras* codon 61 of HT1080 cells, was reported previously and is known to cause activation [25]. The second, a mutation in K-*ras* codon 146 of 8387 (Figure 3), has never been reported in this cell line, or at this location. However, a literature search performed after this mutation was discovered revealed that Hut-14 cells, which are derived from the 8387 cell line [34], contain the same mutation. The gene containing this mutation does not induce focus formation in NIH-3T3 cells, but efficiently transforms an immortal hamster cell line [35]. No other amino acid-altering mutations in any of the three *ras* genes were found among the fibrosarcoma cell lines examined.

Determination of the status of the *ras* genes in cells from the MSU 1.1 lineage:

We also determined the sequences of the coding regions for the Ha-, Ki-, and N-*ras* genes in the cell strains derived from the MSU 1 lineage. No mutations that alter the amino acids encoded by these genes were observed.

The overall frequency of *ras* mutations observed in these two series of cell strains or lines was 2/12 (Table 3). The frequency of *ras* mutations in the cell lines or strains that are capable of forming malignant tumors is 2/8 (Table 3). Therefore, although activation of *ras* by point mutation can be observed in the malignant transformation of human fibroblasts, some other pathway to malignancy must exist.

Table 3. Summary of *ras* gene mutations

Cell group	Mutations/cell line		
	Ha-*ras*	Ki-*ras*	N-*ras*
Fibrosarcoma-derived	0/5	1/5 (8387 codon 146 GCA>CCA Ala>Pro)	1/5 (HT1080 codon 61 CCA>CAA Gln>Lys)
MSU-1 lineage	0/7	0/7	0/7
Total malignant cell lines/strains	0/8	1/8	1/8
Total cell lines/strains	0/12	1/12	1/12

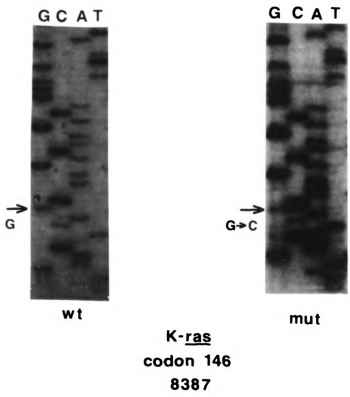

Figure 3. Point mutation in the Ki-*ras* gene of 8387.

This photograph shows the G to C transversion at codon 146 in one allele of the Ki-*ras* gene seen in cells from the 8387 cell line.

DISCUSSION

This study was undertaken to determine the extent to which the malignant transformation of human fibroblasts in vivo and in vitro is associated with the mutational activation of a *ras* gene. Sequencing the coding regions of the Ha-, Ki-, and N-*ras* genes from a total of eight malignant cell strains or cell lines derived from fibrosarcomas revealed only two mutations, one in N-*ras* and one in Ki-*ras*. Mutations were found only in fibrosarcoma-derived cell lines. It is somewhat surprising that none of the malignant transformants within the MSU-1 lineage contained *ras* mutations, especially given the characteristics of these cells. Two of the malignant cell strains are known to have similarly altered p21 immunoprecipitation patterns (unpublished data). A third malignant cell strain forms giant cell tumors very similar to those formed by a malignant derivative of MSU 1.1 that was transformed by an over-expressed and activated N-*ras* gene [16] (unpublished observations). A fourth malignant strain has a typical spindle cell morphology, but its non-tumorigenic precursor has been shown to contain two unique species of phosphoproteins [36]. One of these phosphoproteins is considered to be the platelet-derived growth factor receptor, which is known to stimulate *ras*-mediated signal transduction [37].

The frequency of *ras* mutations seen in our study is similar to that reported in the few studies on *ras* activation and fibroblast transformation. For example, Tahira et al. [38], found Ki-*ras* mutations in cells from one of a total of seven fibrosarcomas from dinitropyrene-treated rats. Similarly, Wilke et al. [39] reported Ha-*ras* mutations in the cells from four of twelve human sarcoma biopsies. These studies show

that there is an association between *ras* activation and malignant transformation of fibroblasts. These correlative data, taken with the numerous studies showing that insertion of activated *ras* transforms fibroblasts in culture, lend support to the hypothesis that *ras* genes can play a role in the malignant transformation of human fibroblasts. However, some other change(s) must also play a role since the majority of fibrosarcomas that were tested did not contain mutated *ras* genes.

It should be pointed out that one of the mutations that we identified was located in a codon that is not one of the "classic" codons typically tested. This shows the importance of determining the sequence of the entire coding region of these genes. This is, of course, a labor-intensive task. However, it is now possible to use single stranded conformation polymorphism to rapidly identify regions in genes where point mutations are located. The advent of such rapid, sensitive techniques makes it feasible to screen the coding region of the three *ras* genes from a large number of cell strains for exons that contain such a mutation and then determine the sequence only of these regions.

A number of investigators have attempted, with varying degrees of success, to establish a cause-effect relationship between *ras* activation and malignant transformation. One study [40] showed an increased frequency of N-*ras* mutations in cells from malignant skin tumors from xeroderma pigmentosum (XP) patients compared to cells from malignant skin tumors from normal patients. XP patients have an inherited defect in the ability to repair DNA damage caused by ultraviolet light and are prone to a high frequency of skin tumors. The N-*ras* mutations found in the cells used in that study were consistent with those typically seen in cells exposed to UV, lending support to a cause-effect relationship between UV

damage and malignant transformation. This cause-effect relationship was called into question by Nakazawa et al.[41]. These investigators tested cells from DMBA-induced BALB/c3T3 foci for an A to T transversion in Ha-*ras* at codon 61 that is commonly found in cells from DMBA-induced tumors. None of the 30 independently cloned focus-forming cell strains had that particular mutation. However, these investigators showed that DMBA can induce A to T mutations in the Ha-*ras* genes of BALB/c3T3 cells at a frequency of 1 in 10^4. These data support these investigors' hypothesis that whether or not a carcinogen-induced mutation contributes to malignant transformation depends on the type of cell in which the mutation occurs. Devereux et al. [42] compared sensitive and resistant strains of mice for the concentration of DNA adducts in lung cells when these mice were treated with two different carcinogens, for the frequency of lung tumors induced by these two carcinogens, and for the frequency of Ki-*ras* activation in the cells from these carcinogen-induced lung tumors. These investigators found large differences both in the frequency of induced tumors and in the frequency of Ki-*ras* mutations between the two strains of mice that could not be explained by the small differences in carcinogen-induced adducts between the two strains. This finding also supports the hypothesis that transformation is affected by factors in addition to carcinogen exposure. Thus, the relationship between carcinogen-induced DNA damage and malignant transformation is complicated by such factors as the ability of the carcinogen to damage DNA, the type of damage done to the target gene, the ability of the cell to repair the damage to this gene before it can be converted into a mutation, and the susceptibility of the cell or tissue to transformation as a result of a mutation in that target gene.

A major difficulty faced by investigators trying to establish a cause-effect relationship between carcinogen-induced *ras* activation and induction of tumors in animals is the relatively high frequency of spontaneous tumors in animal studies [43, 44]. When the frequency of carcinogen-induced tumors is only 2-3 fold above background, interpretation of data is difficult because of the high chance of analyzing spontaneous tumors and attributing the results to carcinogen treatment. An example of spontaneous mutagenesis in the Ha-*ras* gene is that of Hoffmann et al. [45] who showed that mutations at codons 12 and 13 can result when DNA synthesis by DNA polymerase alpha is interrupted by secondary structure of the DNA at that site.

Our finding, and that of others, that activating mutations in *ras* genes occur in some malignant human fibroblasts, leads to speculation about the role, if any, that activated *ras* genes play in the observed malignant transformation. Mutational activation of *ras* genes has been implicated in the acquisition of metastatic capacity by rodent fibroblasts. One study [46] identified an association between expression of activated Ha-*ras* in cultured rat embryo fibroblasts, altered expression of the surface marker CD44 in those cells, and the induction of metastasis by the cells that expressed the altered marker. Studies in our laboratory with human fibrosarcomas also support this association. A series of cell lines or strains that over-express mutated *ras* and form tumors were shown to possess increased secreted and receptor-bound plasminogen activator activity [47] and to exhibit metastatic activity when injected into the tail vein of athymic mice [48]. However, cell strains that did not contain an activated *ras* gene and/or did not over-express *ras* (e.g. HT1080) were found among the cell strains that possess increased

plasminogen activator activity and form metastatic tumors after tail vein injection. Therefore, over-expression of activated *ras* genes is associated with metastasis; however, other changes must also be able to confer this capacity in some cells.

The molecular mechanisms by which *ras* mutation mediates transformation have been the subject of research for many years. Early studies showed that certain nuclear transcriptional factors were required for *ras*-induced transformation [49], indicating the existence of a pathway connecting ras p21 and alterations in gene expression. Recent work has shown that ras p21 cooperates with the product of the *src* gene to transform hamster fibroblasts [50] and human colon epithelial cells [51]. Still more recently research has identified the components of a signal transduction pathway involving *ras* [52-56]. Future investigations should provide insight into the nature of the interaction between these various proteins.

In the cell lines and strains that we showed in the present study did not contain mutations of the *ras* genes, but were malignant, various changes might have occurred. For instance, it has been shown in our laboratory that growth factor expression is altered in several of the fibrosarcoma-derived cell lines [23]. It is also possible that oncogenes unrelated to *ras* have been activated. However, since exogenous activated *ras* genes are efficient transforming agents in fibroblasts, it is possible that the cell lines and strains with only wild-type *ras* genes have changes in other components of the *ras*-mediated signal transduction pathway that are substituting for mutational activation of *ras*. Studies measuring the ratio of GTP-bound and GDP-bound ras p21 are planned, because this measurement will likely provide information that will aid in determining

which, if any, of the ras p21-mediated signal transduction pathway components is activated in these cells.

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APPENDIX

APPENDIX

Recent Developments in *ras* Signalling Pathways

A. Introduction

Evidence has been accumulating since the first discovery of the *ras* genes that the proteins encoded by these genes are critical for regulation of normal growth and for the process of malignant transformation. However, though data linked *ras* activation with transformation, investigators gained little insight into the molecular mechanisms by which ras p21 functions. Early indications that ras p21 functions in a growth factor/mitogenic response pathway came from the data by Mulcahy et al. (1985) who showed that functional p21 is required in order for DNA synthesis to occur, but advances in knowledge about ras p21-mediated signalling pathways have only recently provided information about the role of these pathways in control of growth and cellular responses to extracellular mitogenic signals. A consensus model for the *ras*-mediated signal transduction pathway has been outlined recently in Science (Marx, 1993), as shown in Figure 1. I will summarize these very recent findings, most of which were reported after I had completed the bulk of the research for this dissertation, because this will allow me to place the results I obtained in the context of this new knowledge, and suggest additional experiments that could be done to gain a better understanding of the role of *ras* in normal growth and malignant transformation of human fibroblasts.

B. Intermediates between Growth Factor Receptors and ras p21

The structural properties of ras p21 (attachment to the inner surface of the plasma membrane, guanine nucleotide binding, low level GTPase activity, presence of an effector binding domain) are all consistent with its putative function as a signal transduction molecule. However, it was unclear how p21 is triggered by mitogenic signals such as protein growth factors. Since ras p21 is known to cycle between the active GTP-bound and the inactive GDP-bound forms, it was reasonable to hypothesize that signalling would involve an increase in the GTP-bound form of p21 (Haubruck and McCormick, 1991). Research on signalling mechanisms in several non-mammalian organisms (*Drosophila melanogaster*, *Caenorhabditis elegans*, *Saccharomyces cerevisiae*, *Saccharomyces pombe*), all of which have genes very similar to *ras*, has identified proteins that act as intermediaries between protein growth factor receptors and ras p21. The mammalian counterparts of these intermediary proteins appear to have a similar function (Feig, 1993). At least three related sets of intermediary proteins have been identified. One set appears to be present in numerous cell types, whereas the others are expressed only in specific cell types. Since the ubiquitous set of proteins have been studied the most widely, only these will be reviewed. One of these proteins, encoded in humans by the *hsos1* gene and in rodents by the *msos1* gene, is a guanine nucleotide releasing protein that causes GDP to be released from p21 (Simon et al., 1991; Jones et al., 1991; Bonfini et al., 1992; Bowtell et al., 1992; Wei et al., 1992; Shou et al., 1992). p21 has a much greater affinity for GTP than for GDP. Therefore, once the GDP is released, ras p21 binds to GTP and becomes activated. The other protein, encoded by the *grb2* gene, has no catalytic domain (Clark et al., 1992; Lowenstein et al.,

1992; Simon et al., 1993; Olivier et al., 1993; Buday and Downward, 1993), but does have two types of domains that function as adaptors, i.e., SH2 and SH3. Evidence suggests that the SH2 domain binds to the phosphotyrosine residues of growth factor receptors such as the epidermal growth factor receptor (EGFR), and the SH3 domains interact with the product of the *hsos*1 gene (Rozakis-Adcock et al., 1993).

Evidence for the proposed interactions between growth factor receptors, grb2 adaptor proteins, sos nucleotide exchange factors and ras p21 proteins comes from several sources. Li et al. (1993) showed that when expressed in human kidney 293 cells that have been stimulated with epidermal growth factor (EGF), the human grb2 protein and the hsos1 protein interact. Subsequent work reported from the same laboratory (Gale et al., 1993) using a variant of NIH-3T3 cells that over-expresses the EGFR, showed that an increase in grb2 protein activity results in an increase in the relative amount of GTP-bound p21 (p21-GTP), that this increase is caused by release of GDP rather than by a decrease in GTPase activity, and that the increase in p21-GTP is associated with increased protein kinase activity in the stimulated cells. These investigators also showed that the sos1 protein and the grb2 protein form a complex even in non-stimulated cells, but that the complex associates with the EGFR only when the cells have been stimulated with EGF. Rozakis-Adcock et al. (1993), using a human grb2 protein with a mutated SH3 domain, showed that the SH3 domain is required for grb2 to bind to msos1 protein. Using a similar approach, these investigators also showed that the carboxyl terminal domain of the msos protein binds to grb2. This carboxyl terminal domain has sequence similarity to a protein that binds the SH3 domains of the src and abl proteins. That these intermediary proteins

have been highly conserved across divergent species gives an indication of their importance to normal cell function. In addition, Egan et al. (1993) showed that over-expression of the *Drosophila* sos1 protein can transform NIH-3T3 cells, lending support to the hypothesis that this part of the signal transduction pathway is also important to malignant transformation.

C. Intermediates between ras p21-GTP and the Nucleus

1. Intermediates that Appear to Interact Directly with p21-GTP

Events that occur after ras p21 has bound GTP have also been the subject of extensive research. The consensus of these investigations is that the interaction between p21-GTP and one or more cytoplasmic protein kinases initiates a phosphorylation cascade that results in the activation of nuclear transcription factors and triggers expression of the genes that control mitogenesis. The following paragraphs describe some examples of research in this area.

Morrison et al. (1988) showed that transformation of NIH-3T3 cells with oncogenes encoding membrane-bound proteins (*src*, *fms*, *sis*, *ras*, and polyoma *middle T*) resulted in cells capable of phosphorylating the raf-1 protein, a serine/threonine kinase that is the product of the *raf*-1 gene, the mammalian homologue of the v-*raf* oncogene of the murine sarcoma virus 3611 (Rapp et al., 1983). This led to the hypothesis that the raf-1 protein interacts with ras p21 and other membrane-bound oncogene products as part of a signal transduction pathway. However, Reed et al. (1991) failed to observe phosphorylation of the raf-1 protein in response to expression of activated H-*ras* in NIH-3T3 cells unless the cells had also been stimulated with a protein growth factor. In their study, cells that

expressed the v-src protein did not require additional growth factor stimulation to phosphorylate the raf-1 protein. This led the authors to conclude that an activated ras protein, unlike the src protein, cannot initiate a mitogenic signal, but can only amplify signals provided by protein growth factors. However, it should be noted that although the cells used in this study by Reed et al. (1991) had been transformed by an activated *ras* gene, they were not able to cycle without the addition of exogenous growth factors. In many other experimental transformation systems, cells transformed by transfection of *ras* are growth factor independent, and in some cases are fully transformed. One explanation for the results of Reed et al. (1991) is that in their system, some component of the ras p21-mediated pathway prior to the raf-1 protein was not functioning.

Another approach to determining whether there is interaction between ras p21 and the protein kinases in the signal transduction pathway, is to transfect cells with a gene encoding a dominant-negative mutant of ras p21 and to observe the effect that mutant ras p21 has on the phosphorylation state and/or the kinase activity of the protein kinases. An example of this approach is the work of Wood et al. (1992) who transformed human pheochromocytoma cells with a dominant-negative *ras* mutant whose expression could be controlled with dexamethasone. Expression of this non-functioning mutant p21 abolished the phosphorylation of the raf-1 protein and other cytosolic protein kinases that normally occurs in response to stimulation with protein growth factors. This indicates that the presence of functioning ras p21 is critical for transmission of the mitogenic signal. The block was not always complete, but whether this reflected the presence of a *ras*-independent pathway or merely the

inability of mutant ras p21 to fully compete with endogenous ras p21 was not determined. Wood et al. (1992) also showed that increased phosphorylation of the raf-1 protein occurred when the cells expressed an oncogenic form of *ras*. Thomas et al. (1992) used this same approach to show that functional ras p21 is required to stimulate the activity of another serine/threonine kinase, mitogen-activated protein kinase (MAPK). These investigators also showed that the ras p21-induced increase in MAPK activity resulted from phosphorylation of MAPK, not from its over-expression.

Work by Warne et al. (1993) also linked phosphorylation of the raf-1 protein to interaction with p21-GTP. In addition, they showed that the effector domain of ras p21 interacts with the amino terminal domain of the raf-1 protein. Moodie et al. (1993) studied the association between ras p21 and the raf-1 protein using an in vitro system in which ras p21 immobilized on silicon beads was used to probe rat brain cytosol extracts. These investigators showed that wild-type p21-GTP strongly bound the raf-1 protein and stimulated its kinase activity whereas wild-type p21-GDP bound raf-1 poorly and did not stimulate its kinase activity. Interestingly, the GDP-bound form of an oncogenic variant of ras p21, with valine instead of glycine at codon 12, bound poorly to the raf-1 protein but nonetheless strongly stimulated raf-1 protein kinase activity. This led to the hypothesis that oncogenic ras p21 has an altered structure that allows constitutive interaction with the raf-1 protein or with other intermediates in the pathway.

2. An Intermediate that Interacts Directly with the raf-1 protein

A study providing strong evidence for the interaction between Raf1 and another protein in the chain, MAPK kinase (MAPKK), was reported by Hughes et al. (1993). Since MAPKK has a high degree of sequence similarity to the *Saccharomyces pombe* mating factor encoded by the *byr*1 gene, these investigators attempted to complement a *byr*1-minus mutant with the mammalian MAPKK protein. They could not successfully complement the mutant unless the raf-1 protein was expressed along with MAPKK. By mixing cellular extracts and testing for MAPKK activity they showed that its kinase activity was dependent on the raf-1 protein. Enhancement of MAPKK activity was the result of increased phosphorylation of MAPKK, not increased protein expression.

D. Conclusions

Several problems in the ras p21-mediated signalling pathway remain unresolved. One example is that the specific reactions between the intermediates in the pathway, the kinds of control mechanisms that are present, and the interactions, if any, between various pathways are not yet defined. A second example is that the role in this pathway of ras p21 itself needs further clarification, for instance, the differences in wild-type and activated ras p21 and the proteins with which they interact. Studies to resolve these problems must be carried out with attention to cell biology, in particular to the ability of the cell to divide under the conditions of the experiment, and to the transformation status of the cells. The results to date of studies that I have recently been carrying out indicate that the cycling characteristics of a cell line are closely related to its ras p21 GTP/GDP ratio, to the nature of its *ras* genes, and

to the level of expression of its *ras* genes. However, the precise relationship of these variables to one another and to cell transformation is not entirely clear. Experiments are planned to determine whether the cell lines and strains that I showed do not have an activated *ras* gene do have an alteration in one or more of the other components in the *ras*-mediated pathway that is contributing to transformation. One approach will be to determine the p21 GTP/GDP ratio in cells that contain two wild-type *ras* alleles. It is possible that alterations in either *grb2* or *sos1* result in constitutive activation of p21, and if so, this would be expected to result in an increase in GTP-bound ras p21. in contrast, alteration of one of the protein kinases that normally interacts with ras p21-GTP would not be expected to be reflected in an increase in GTP-bound ras p21. Such information, along with what is known about *ras* genes from research in this laboratory and from data in the literature on the *ras*-mediated signalling pathways, should yield insights into malignant transformation of human fibroblasts.

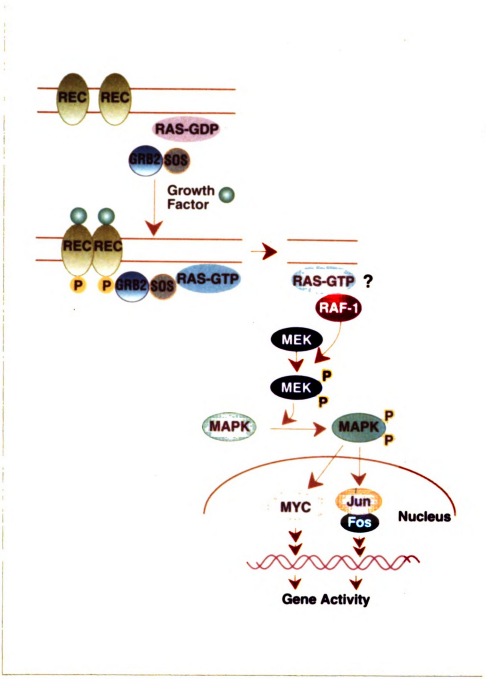

Figure 1. Consensus model of the *ras*-mediated signalling pathway (Reprinted with permission of AAAS. Marx, J. Forging a path to the nucleus [Research News]. Science 260:1589, 6/11/93, ©AAAS.)

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