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Viral jun oncogene serves as a suppressor of phorbol ester TPA induced tumor cell invasion and stromelysin gene expression

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The University of Arizona, 1994
VIRAL JUN ONCOGENE SERVES AS A SUPPRESSER OF PHORBOL ESTER TPA INDUCED TUMOR CELL INVASION AND STROMELYSIN GENE EXPRESSION

by

Tom Chun-Chang Tsang

A Dissertation Submitted to the Faculty of the DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY In Partial Fulfillment of the Requirements For the Degree of DOCTOR OF PHILOSOPHY In the Graduate College THE UNIVERSITY OF ARIZONA 1994
As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Tom Chun-Chang Tsang entitled Viral Jun Oncogene Serves as A Suppressor of Phorbol Ester TPA Induced Tumor Cell Invasion and Stromelysin Gene Expression and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

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

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

G. Tim Bowden
Dissertation Director
Statement by the author

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Signed: Tom Chun-Chang Tsang
Acknowledgments:

I would like to thank my family, my teachers, all of my friends and colleagues for helping me through these years. I would like to especially thank Joanne because she is all of the above to me.
Dedication:

I would like to dedicate this work to my aunt Tsun-Tao Chen, who died of breast cancer last year.
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Abstract

Carcinogenesis in the mouse skin is a multistep process involving initiation, promotion, and progression. In order for the benign papilloma cells that have been initiated and promoted to become malignant, genetic alterations are believed to be involved.

The viral jun (v-jun) oncogene encodes a transcription factor that can participate in the transactivation of genes through the AP-1 complex. Evidence indicates that the ability of v-jun to transform cells and stimulate transcription depends on the cell type.

The question that I have attempted to answer in my dissertation studies is whether expression of the v-jun gene in benign tumor forming mouse keratinocytes that already express an activated c-rasHa oncogene would cause malignant progression. Our results showed that the v-jun transfection did not result in malignant progression; instead, we made the unexpected observation that the ability of these cells to invade reconstituted basement membrane matrix (in vitro) in response to the phorbol ester, 12-0-tetradecanoyl-phorbol-13-acetate (TPA) was suppressed.

This phenomenon could partially be explained by the suppression of the induction by phorbol ester of expression of the metalloproteinase, stromelysin (transin). Of interest
was the finding that TPA induction of other cellular genes known to be regulated by AP-1 was not inhibited in the benign tumor cells expressing v-jun.

The suppressor activity shown by v-jun is different than that of other tumor suppressor genes in that it appears to be specific for the process of tumor invasion. A potential implication of this unexpected finding is that it may provide insight on how to develop cancer therapeutic strategies that can specifically inhibit tumor invasion.

I have constructed a set of cassette cloning vectors that can be used for rapid adaptation of DNA restriction fragments. In addition, I have formulated a new model with regards to the mechanism of function for the 70 kDa family of heat shock proteins (hsp 70). This model proposes that hsp 70 serves as a cross-linker molecule between many cellular proteins and the cytoskeletal matrix, and this model may have significant implications on many cellular processes.
An overview of cancer

"Cancer" is defined as "A disease characterized by proliferation of cells leading to local growth (i.e., a tumor), to local invasion, and to metastasis." (Hill et al. 1987). The term "cancer" comes from the Latin word "cancer" and the Greek word "karkinos", both meaning "crab" (Tomatis, 1990). This describes the fact that some forms of malignant tumors have the appearance of the crab with a central core and leg-like extensions that spread into surrounding tissues.

The first known reference describing a disease which was most likely cancer occurred about 5,000 years ago (Shimkin, 1977). Cancer has been found in all animal species that have been closely examined (Tomatis, 1990). In fact, evidence found in the fossil records of a dinosaur that lived millions of years ago suggested that it suffered from osteoma and haemangioma (Tomatis, 1990).

In the past one hundred years, numerous medical advancements, especially the great success in controlling the various communicative diseases and the discovery of the antibiotics, have lead to a dramatic increase in the life expectancy of the world population. As the life expectancy continues to rise, cancer becomes a greater health problem.
In 1992, there were more than 1.13 million cases of newly diagnosed cancers in the United States, and over 520,000 people died of cancer (Boring et al. 1992). The number of deaths caused by cancer is over 7 million worldwide. All types of cancer combined is currently the second leading cause of death in United States and if the current trend continues, cancer will replace cardiovascular disease as the leading cause of death by the year 2000 (Boring et al. 1992).

These grim statistics do not begin to tell the human tragedies that cancer causes. All of us have been effected in some way by cancer. This disease generally lasts for years and the treatments are often more painful and disabilitating than the disease itself. The emotional suffering and the economical burden on both the patient and their families makes cancer perhaps one of the most feared diseases of the modern world.

Carcinogenesis

There are a large variety of agents that have been shown to cause cancer. These can basically be classified into three major groups, chemical, physical and viral. In addition, there are a diverse set of agents that are generally not carcinogens by themselves but can greatly increase the
incidence and shorten the latency of tumors in experimental models (Boutwell, 1985; Yuspa, 1986). These are called tumor promoters and they are often agents that can induce the proliferation of the target cells.

Chemical carcinogens are identified with the help of epidemiological data and experimental animal studies. Many of these chemicals are present in our diet (Ames, 1983). There are also a large number of chemical carcinogens from industrial, medical and social sources. Some of the chemicals found in the environment, such as aflatoxin B1, N-nitrosamides and N-nitrosamines are also thought to be cancer causing agents (Slaga, 1991).

Chemical carcinogenesis often involves metabolic activation. The Millers have proposed a unifying general theory to explain the mechanism of action of chemical carcinogens (Miller et al., 1976). It stated that all chemical carcinogens that are not electrophilic agents must be metabolically converted into a electrophilic form that reacts with some nucleophilic sites in either DNA or other macromolecules.

The compounds in this class that were believed to be highly significant in human cancers are the polycyclic aromatic hydrocarbons, aromatic amines, and nitrosamines (Slaga, 1991). All of these carcinogens can potentially lead to the formation of the DNA adducts that can cause the
mispairing of the DNA bases. After subsequent rounds of DNA replication, mutations will have occurred (Slaga, 1991).

A number of physical agents have also been shown to cause cancer. These included ultraviolet light, asbestos, and ionizing radiation (Rauth, 1991). Less than ten years after its discovery by Roentgen, X-rays were reported to be carcinogenic (Tomatis, 1990). Ionizing radiation is the best characterized of these agents. There are many epidemiological and experimental animal studies related to the molecular mechanisms involved (Jaffe et al. 1986; Rauth, 1991).

When a photon strikes an electron and the energy of the photon exceeds that of the binding energy between that electron with its atoms or molecules, it can cause the electron to be removed from the target atom or molecule. This is known as the ionization event (Weichselbaum et al., 1993). An ionized particle is unstable. It can react very quickly with a target molecule and causes a change in the chemical structure of the target molecule (Weichselbaum et al., 1993).

A direct hit by radiation on the sugar phosphate backbone of DNA can cause both single and double strand breaks. When the photon interacts with water molecule, it can produce both hydrogen and hydroxyl radicals (Weichselbaum et al., 1993). When these free radicals interact with DNA, they can also cause DNA strand breaks or change the structure of the bases in such a way that it leads to mispairing of the
base pairs (Weichselbaum et al., 1993).

Viruses can also cause cancer. The first evidence linking viral infection to cancer was presented by Peyton Rous (Tomatis, 1990). He showed that filtered extracts from chicken fibrosarcomas can infect the recipient chickens and produce fibrosarcomas. It is now known that this virus, the Rous sarcoma virus (RSV), is a retrovirus (Tomatis, 1990).

Carcinogenesis by retroviruses can basically be divided into two modes of infection (Sheinin et al. 1987; Minden, 1987). The acute transforming retroviruses generally contain genes that can cause cancer and are called oncogenes. The slow transforming retroviruses can cause cancer through the mechanism of insertional mutagenesis (Sheinin et al. 1987; Minden, 1987). Viral insertion can also lead to an alteration in the regulation of cellular genes and therefore causes transformation.

Although it now appears that viral infections have only played a minor role in the etiology of human cancers, viruses have been an extremely important tool in the studies of cancers (Sheinin et al. 1987; Minden, 1987). Through studying viruses both as a cancer causing and cancer contributing agents, researchers have made many breakthroughs in our understanding of the normal and pathological cellular functions. Much of what we know today about cellular oncogenes and tumor suppressor genes come from studies of
viral oncogene products (Sheinin et al. 1987; Minden, 1987).

In view of the fact that we are constantly exposed to all these cancer causing agents, it is perhaps more remarkable that we don't all develop cancer at an early age. The fact that our body's own natural defenses such as the immune system is perhaps millions of times more effective than any therapeutic strategies that we have developed should be something that all cancer researchers be frequently reminded of. Such ideas have led to some of the new therapeutic approaches using the components of the immune system and endocrine system.

Cancer therapies

There are certain principles that applied to the treatment of all diseases (Holland et al., 1993). These principles were clearly stated by the prominent medical professor Dr. Robert F. Loeb more than 50 years ago, and today, they are known collectively as Loeb's Laws of Therapeutics (Holland et al., 1993). They are:

1. If what you are doing is doing good, keep doing it.
2. If what you are doing is not doing good, stop doing it.
3. If you don't know what to do, do nothing.
4. Never make the treatment worse than the disease.
Cancer therapy represents one of the most complex problems in medicine and currently, many treatments do not follow the basic laws of therapeutics. The difficulty in dealing with cancer in part comes from the fact that cancer is not one disease, but instead many different diseases with different causes and perhaps different treatments (Harris, 1991).

Tumors can develop in almost every organ of the body and from almost every cell type in that particular organ. Even among tumors originating from the same cell type in the same organ, there are enormous variations between the individual patients in terms of the tumor's genetic make up, the morphology of the tumor as well as in their responsiveness to a particular treatment (Harris, 1991).

Furthermore, although evidence has indicated that tumors are derived from a single cell (Nowell, 1976), a tumor is composed of a heterogeneous population of cells (Wainscoat et al. 1990). This is because tumor development is composed of many mini-evolutionary events. Depending on such variables like the stage in tumor's progression, the immediate local environment of the tumor and interactions with the immune system, different populations are selected. By the time a single cancer cell has completed the forty or so doublings to become a clinically detectable tumor, there are already many subpopulations (Nowell, 1976; Wainscoat et al. 1990). Thus,
it is possible that no two cases of cancer are exactly alike. This also means that there may never be a single cure for cancer, but instead, each type of cancer may require a specific or a combination of therapeutic strategies.

Traditional therapy for cancer can basically be divided into three categories: surgery, radiation therapy and chemotherapy. All of these therapies have been very successful in treating some types of cancers but each have some important limitations.

Surgery is the oldest and the most often used therapy of cancer (Morton, 1993). Although today it is rarely used alone, surgery is also considered the single most effective cure of cancer. In 1809, before the discovery of the antiseptics and anesthesia, an American surgeon named Ephraim MacDowell, performed the first recorded successful treatment of a tumor by surgery (Morton, 1993). He removed a twenty-two pound tumor from the ovary of a woman. This patient then went on to live for another 30 years.

The current ideas regarding surgery is that it is a local therapy. Surgical procedures are designed to remove the primary tumor and those tumor cells that have followed the usual route of spread such as the regional lymph nodes (Morton, 1993). This type of logic would suggest that once a tumor has spread to a distant site, surgery is no longer effective as a treatment. Nevertheless, some evidence has
indicated that some patients can be cured of cancer by local excision of the primary tumor even though there is evidence that the tumor has already metastasized (Morton, 1993).

It is thought perhaps surgery of the tumor mass can be considered to be a form of immunotherapy. Evidence has been shown that tumor cells are often secreting factors that are capable of inhibiting the effectiveness of the immune system (Hellstrom, 1974). By removing the tumor cell mass that is producing large quantities of general immunosuppressive factors, one can effectively revitalize the immune system and increase its efficiency in destroying small distant metastatic colonies of tumor cells (Morton, 1993).

X-ray was discovered by Roentgen in 1895, and one year later, gamma rays were discovered by the Curies. It was known by 1902 not only that radiation can be carcinogenic but also that it can have harmful effects on both normal and tumor tissues (Weichselbaum et al., 1993). The use of radiation as a clinical treatment for laryngeal carcinoma began in the 1920's, and the development of Cobalt-60 sources and linear accelerators in the 1950's allowed the treatment of deep tumors with high dose radiation (Weichselbaum et al., 1993).

The main target molecule for radiation therapy is DNA (Weichselbaum et al., 1993). Cell killing is usually achieved after several more rounds of replication due to extensive damage to the DNA. The fact that cell death may not occur
immediately after irradiation could have significant clinical implications. Some slow growing tumors are not very sensitive to radiation treatment. Studies have shown that it takes up to 24 months after irradiation for prostate adenocarcinomas to show any histological alterations (Cox et al., 1983).

Radiation is most effective for treating fast dividing tumor cells. It also shows the most severe toxic side effect for rapidly dividing normal cells. A constant fraction of cells are killed at a given treatment dosage (Weichselbaum et al., 1993). Therefore, radiation treatment could potentially select for a radiation resistant population. This is consistent with the finding that the intrinsic resistance of tumor cells to ionizing radiation seemed to increase for cells as they become tumorigenic (Sklar, 1988).

Chemotherapy has been used since 1940's for treating cancer. In general, it is a systemic treatment that targets the mitosis of the cells (Norton et al. 1993). Consequently, it is most effective against rapidly proliferating tumors and it is most toxic to dividing host cell type such as hair follicles, gastrointestinal mucosa and bone marrow cells.

Like radiation, chemotherapy has a tendency to select for tumor populations that are resistant to the treatment (Morrow et al., 1993). Often, the tumor will become resistant to multiple drugs at the same time. Furthermore, experimental evidence have long shown that some chemotherapeutic agents
are also carcinogens (Sieber et al., 1975).

Therefore, an on-going fear of using chemotherapy is that it can cause new cancers to develop. These concerns were shown to be justified when it was discovered in clinical trials that patients who were treated for ovarian cancer with alkylating agents have a significantly higher rate of developing acute non-lymphocytic leukemia (Greene et al., 1982).

As mentioned earlier, one of the mechanisms whereby surgery could be successful in curing cancer is thought to be caused by a reduction of the overall tumor mass which leads to a significant reduction of the immunosuppressive factors secreted by the tumor.

Unfortunately, in both radiation therapy and chemotherapy, the immune system cells are among the host cells that are most susceptible to the toxic side effects of the treatment. This means that after radiation and chemotherapy, the immune system may be much less effective in fighting the micro-tumor colonies that have already metastasized.

These therapeutic strategies are often used in combination and together they can cure about half of all cancer cases (Liotta, 1985). However, evidence has suggested that for some types of cancers such as prostate cancer, there does not appear to be any significant improvement in the
patient's survival after therapy.

The ultimate challenge for cancer researcher is not only to find ways of improving on the current therapeutic strategies but also to develop more effective new strategies. By improving our understanding for the biological basis of cancer, we can potentially discover some exploitable differences between cancer cells and normal cells which may lead to novel therapies for cancer.

**Cancer Biology**

Compared to the studies of cancer causing agents and cancer therapies, it was only relatively recently that we have begun to understand the molecular and cellular basis of cancer. It is now firmly established that cancer is caused by mutations in the DNA that lead to alterations in the normal cellular regulation and function (Tannock et al. 1987).

One of the defining characteristics for cancer cells is that they do not respond normally to controlling signals for growth and differentiation. Cancer cells are not only immortal, they also can proliferate indefinitely, but sometimes, this proliferation occurs very slowly, even slower than normal cells. The other defining characteristics for malignant cancer cells is that they invade surrounding tissues and they have the potential for metastasis (Kohn et
al., 1991).

These two characteristics have both separate as well as overlapping features. Uncontrolled growth by itself does not cause invasion and metastasis. These two aspects of cancer cells are often studied using different experimental approaches, and theoretically, they can each provide independent targets for therapeutic interventions.

It should be emphasized that although it is generally the invasive and metastatic properties of the cancer cells that eventually cause the death of the patient, both radiation therapy and chemotherapy primarily target the proliferation of the cells.

Early in the development of the mammalian embryos, up to the blastocyst stage, all cells are functionally interchangeable. These cells are not only undergoing rapid division, they also have the potential to divide indefinitely; they are immortal (Alberts et al., 1983). After gastrulation, the cells in the embryo begin the process of differentiation by acquiring distinct characteristics, and they eventually become differentiated into one of the approximately 200 histologically distinguishable cell types in the mammal.

The processes of cell growth and differentiation are under very strict control. In adult animals, there are both fully differentiated cells as well as less differentiated
stem cells (Alberts et al., 1983). Cell proliferation is dependent on highly coordinated interactions between various cells and cell types in a particular environment. Generally, it does not occur without the stimulation by specific growth factors.

The growth factors bind to specific cell surface receptors and this activates a signal transduction pathway that relays the signal from the cell surface to the nucleus. Upon receiving the signal, a set of DNA binding proteins known as the transcription factors will then activate the transcription of various growth related genes by binding to the regulatory regions of these genes. This process may also involve inactivating a suppressor of transcription.

Oncogenes were originally identified as the transforming genes carried by tumor causing retroviruses (Bishop, 1987). It was later discovered that there are cellular homologues to these genes called proto-oncogenes (Bishop, 1987). Nearly all of the oncogenes and proto-oncogenes coded for products that functioned somewhere along the signal transduction pathway. These included the growth factors, membrane receptors, non-receptor kinases, signal transducers, transcription factors and nuclear proteins (Bishop, 1987).

The retroviral oncogenes are all different from their cellular counterpart in that they are "activated" by mutations in the coding sequences resulting in an altered
gene product or in promoter regions leading to an increase in their level of expression (Minden, 1987). The common end result of retroviral oncogene insertion is that the infected cells act as if they are continuously being stimulated to proliferate.

There is a significant amount of data now indicating that the inactivation of tumor suppressor genes may be as important in cancer development as oncogene activation (Fearon et al., 1993). These data come from studies of epidemiological patterns of some cancers that followed the patterns of an inherited traits, somatic cell hybridization studies and analysis of the non-random patterns of chromosome losses during tumor cell development.

The study of the tumor suppressor genes is one of the most active and exciting fields in cancer research today. It was thought that the number of potential suppressor genes may be almost as large as the number of oncogenes (Fearon et al., 1993). Many of these tumor suppressor genes have now been isolated and some link has been established in their loss of function with the occurrence of human tumors (Fearon et al., 1990).

So far, however, the normal cellular functions of these suppressor genes are not fully understood. One hypothesis suggested that some of them may function as transcriptional suppressors, perhaps by binding to specific DNA regulatory
sequences (Kern et al., 1991).

In principle, the loss of function in either the negative regulatory genes in the signal transduction pathway for cell proliferation or any of the genes that functioned in the induction and the maintenance of the cells in a differentiated state can give the tumor cells a selective advantage. These losses could cause a cell to become immortalized or it could induce the cells to proliferate without control. Thus, all these genes are candidates for potential tumor suppressor genes.

At least two other mechanisms that can potentially be played by tumor suppressor genes but have not received much attention are the maintenance of genomic integrity and interaction with the immune surveillance system.

Because cancer is a multistep processes that requires several mutations, any loss of function in the cellular mechanisms for preventing mutations will increase the probability of developing cancer. This would suggest that mutations such as those that decreased the fidelity of DNA replication, decreased the efficiency of the DNA repair systems and decreased the structural integrity of the chromosomes, could all contribute to carcinogenesis.

Cells are constantly processing and presenting antigens onto their surface for immune surveillance purposes. The cell is targeted for destruction if the T lymphocytes recognize
the antigen to be foreign or abnormal. In theory, this is one of the mechanisms by which cancer cells are detected and destroyed by the immune system. If a mutation causes the cellular machinery for antigen presentation to malfunction, it is possible that a transforming mutation within the cells will result in the cell no longer being monitored by immune surveillance. A loss of function in the antigen presenting pathway may also play a role in the development of cancer.

The characteristic that generally makes cancerous cells lethal to their host is that they invade surrounding tissues and also metastasize. This may require further genetic alterations. Invasion and metastasis both involve a group of highly coordinated processes. It can be subdivided into several steps (Liotta et al., 1991). First, the tumor cell must leave the primary tumor. This requires cell locomotion. Next, it must attach to the host extracellular matrix components and invade the local host tissue. This involves the proteolytic degradation of such physical barriers as the basement membrane. After the tumor cell gains entrance into the circulation and travels to a distant site, it must be able to re-attach itself to the extracellular matrix of another organ. Then, the tumor cells must re-invade this target organ and gain access to nourishment, and finally, proliferate as a secondary colony.

All of these steps must be highly coordinated (Liotta et
al., 1991). For example, although the functions of proteolytic enzymes are necessary for the degradation of the basement membrane, a metastatic tumor cell can not simply indiscriminately produce and secrete large amount of protein degrading enzymes. This is because cell locomotion during invasion requires the orderly attachment and detachment of the cells as they move. Degradation of all surrounding matrix proteins would in effect destroy the tracts on which the cells are required to move (Liotta et al., 1991).

Tumor cell invasion and metastasis may be achieved by fraudulently turning on a cascade of normal physiological processes that involve cellular locomotion and invasion, such as embryogenesis, wound healing tissue remodeling, angiogenesis, and parasitic or bacterial infection (Liotta et al., 1991).

The ultimate goal of studying genes involved in cancer is to apply this knowledge in cancer diagnostics, prevention and therapy. Recent developments in the new field of gene therapy have brought this closer to reality (Rosenberg, 1991). The basic concept of gene therapy is to re-introduce genes into target cells and therefore change the phenotype of these cells. This strategy is currently being used to re-introduce normal enzymatic functions into the cells of patients with genetic metabolic deficiencies (Rosenberg, 1992).
In principle, it is possible to revert a cancer cell that has lost its suppressor genes back to a normal cell by re-introducing the particular tumor suppressor gene(s). Several labs have already shown that replacing the missing suppressor gene can abolish the tumor forming ability of some cell lines (Huang, 1988; Chen, 1990; Baker, 1990).

There are severe limitations to this approach in treating human cancer. The greatest technical challenge is perhaps the efficiency of the delivery: how to get the treatment to every tumor cell? This is a critical question because even if only one tumor cell escapes treatment, theoretically there is a risk of cancer reappearing. Currently, there are no methods available that can achieve 100% tumor cell killing in vivo.

The second limitation is the specificity of the delivery method. Since virtually all oncogenes and suppressor genes function in some aspect of normal cellular processes, it can be expected that any alteration of their function will be detrimental to the normal cell. Thus, any method of delivery that is not specific for the tumor cells will have toxic side effects if the normal cellular processes of the non-tumorigenic cells are also effected. An additional problem is the selection of the resistant population of tumor cells. This might occur because constitutively over-expressing tumor suppressor genes generally inhibits the growth of the tumor
cells (Huang, 1988; Baker, 1990), there will be selective pressure placed on the population of tumor cells to become resistant to treatment. Since cancer cells can evolve at a much faster rate than normal cells due to the instability of their genome (Harris, 1991), this is perhaps the ultimate limitation of the gene therapy approach. Thus, the current long term prospect for curing cancer by replacement of the tumor suppressor genes may not be more promising than either radiation therapy or chemotherapy.

An alternative approach to cancer therapy is to target processes in tumor development that are involved in tumor cell invasion and metastasis (Liotta et al. 1991). Such strategies have a great deal of potential because the majority of the deaths caused by cancer is due to metastatic lesions (Liotta et al. 1991).

In principle, if one can specifically inhibit tumor cell invasion but not its growth, there will be less toxicity and there will be less selective pressure placed on the tumor population to evolve a resistance to the treatment. By controlling the invasion and therefore the spread of cancer, one can improve the efficiency of other treatments that could be used in combination.

**Experimental systems**
The goal of my research was to gain further understanding of the events involved in the process of tumor cell invasion. This knowledge could potentially lead to therapeutic approaches that could target the processes of tumor cell invasion.

Due to the extraordinary complexity of human cancer, virtually all cancer research experiments are performed using greatly simplified experimental systems. This is necessary in order to reduce the number of variables so that clean and reproducible data can be produced. The obvious risk in this approach is in over-simplifying the experimental system so much that the results are no longer relevant to the biological problem that we are trying to study. Thus, a decision that a student of cancer research must learn to make is how to choose experiments that will generate clean and reproducible data and at the same time may be relevant in the struggle to end the suffering caused by human cancer.

The experimental system that we have chosen is the mouse multistage skin carcinogenesis model. This system has been used extensively in the past and much useful information has been accumulated (Slaga, 1991). There are some important advantages that this experimental model can offer in the study of cancer. Although cancer can develop from many cell types, most clinically reported human cancers are derived
from a single cell type, the epithelium. The epithelium is the layer of tissue that covers the body and makes up the skin, and it is also the cell type that lines or covers all organs. Cancer derived from the epithelium are called carcinomas (Hill et al. 1987). These include such common cancers as lung, breast, skin, prostate, cervix and colon.

The epithelial cells are separated from the rest of the body by a physical barrier, the basement membrane, also known as the basal lamina (Liotta et al. 1991). The basement membrane is composed of many proteins that make up the extracellular matrix. These proteins included collagen, gelatin, laminin, entactin, proteoglycan and fibronectin (Liotta et al. 1991). Together these proteins form an intricate matrix and their complex physiological roles are just beginning to be understood at the molecular level.

The carcinogenesis of the epithelial cells has been experimentally defined as three stages, initiation, promotion and progression (Slaga, 1991; Liotta et al. 1991) (Fig. 1). Each of these stages has distinct genetic and phenotypic characteristics. In the murine epithelial system, each of these stages have been isolated. There are many well-characterized murine keratinocyte cell-lines that are available for various manipulations in vitro (Strickland,
Fig. 1 Mouse skin multistage carcinogenesis model.
The initiation stage involves a somatic mutation in some aspect of epidermal growth control and/or epidermal differentiation and it is irreversible (Slaga, 1991; Liotta et al. 1991). This is generally caused by exposure to subcarcinogenic dose of complete carcinogens. Initiation alone does not cause tumors. Current evidence has indicated that initiation does not necessarily cause any change in the rate of DNA synthesis, cell division or any change in the enzymatic activities of the cells (Boutwell, 1985).

Promotion involves the multiple applications of a group of agents called tumor promoters. These are generally not carcinogens but they have many different effects on the cells treated, including induced DNA synthesis, cell division and many changes in the morphological characteristics of the cells (Boutwell, 1985).

An important difference between mechanisms responsible for initiation and promotion events is that effects of the promoting agents are reversible. To get the greatest effect, multiple rounds of the promoting agent must be applied for a long time in short intervals (Boutwell, 1964).

In mice treated with either a subcarcinogenic dose of initiating agent or multiple doses of a promoting agent, no tumors are formed. However, many tumor will form if the mice are first treated with the initiating agent and followed with
the promoting agent (Boutwell, 1964 and 1974). Interestingly, the time lag between initiation stage and the beginning of promotion stage does not appear to be important (Van Duuren et al., 1975), but tumors will not form if the order of treatment is reversed (Roe, 1959).

Some investigators have suggested that perhaps the ultimate determining factor in the development of many human cancers is the level of promoting agents to which one is exposed (Boutwell, 1985). This is because humans are inevitably exposed to a significant level of initiating agents (Rauth, 1991; Ames, 1983). Initiation, therefore, may be a frequent event.

Many tumor promoting agents are produced endogenously. Some examples are wound hormones, growth hormones, polypeptide hormones, anabolic steroids, bile salts, and abnormal amino acid metabolites (Boutwell, 1985). The best characterized of promoting agent is the phorbol ester 12-O-tetradecanoyl phorbol-13-acetate (TPA).

TPA is an extremely potent tumor promoter in the mouse multistage skin carcinogenesis model. It can induce a transient malignant phenotype as well as stimulate the growth of the cells (Boutwell, 1985). Treatment with TPA also induces a change in the regulation of many cellular genes. These genes can play a functional role in both the growth of the cells and in the processes of invasion.
Most of the tumors induced by the initiation-promotion protocols are benign papillomas. The third stage of the multistage model involves the progression of these benign papillomas to malignant carcinomas. Current data indicate that this may require another mutational event (Slaga, 1991).

Hennings and colleagues have discovered that tumor progression is independent of treatment with tumor promoter but an additional application of an initiating agent increased the conversion of benign papillomas to malignant carcinomas (Hennings et al., 1983). In addition, it was shown that ionizing radiation can also increase the rate of conversion of chemically induced benign papillomas (Jaffe et al., 1986 and 1989), thus providing further evidence that new genetic alterations are involved in the progression of benign papillomas to invasive carcinomas.

There is now a considerable amount of evidence indicating that the activation of the c-ras$^{Ha}$ oncogene plays a crucial role in the early initiation stages of the mouse skin multistage carcinogenesis model (Balmain et al., 1983; 1984; Roop et al., 1986). It was shown that a majority of both benign and malignant tumors induced in the mouse skin using a potent initiating agent 7, 12-dimethylbenz (a) anthracene (DMBA) contained an activating mutation in the 61st codon of c-ras$^{Ha}$ oncogene (Balmain et al., 1983; 1984).

Studies using a mouse squamous papilloma cell line that
contained an activated c-ras$^{Ha}$ oncogene, 308, have shown that transfection with the v-fos oncogene can complement activated c-ras$^{Ha}$ to cause malignant conversion of 308 cells (Greenhalgh et al., 1990). Furthermore, it was shown in the nude mouse skin graft assay that two oncogenes, v-fos and v-ras can cooperate to convert normal mouse keratinocytes into squamous cell carcinomas (Greenhalgh et al., 1990).

Recently, it was shown that transgenic mice that express both v-fos and v-ras can cooperate to induce papillomas with an autonomous growth phenotype (Greenhalgh et al., 1993). However, contrary to the authors' previous conclusions (Greenhalgh et al., 1990), they did not detect malignant squamous cell carcinomas. Instead, these mice showed a greater severity in preneoplastic phenotypes and earlier formation of papillomas as compared to transgenic mice expressing either v-ras or v-fos alone.

These researchers speculated that the reason that no malignant tumors had developed was due to the intrinsic differences between the two experimental systems (Greenhalgh et al., 1993). Specifically, the fact that nude mice are immune deficient and that wounding inevitably occurs during the skin graft assays may have contributed to tumor progression. Based on these new data, they suggested that additional events may be required for malignant conversion (Greenhalgh et al., 1993).
Experimental Approaches

There are basically two different experimental approaches to study the genetic events during progression. The first is the endogenous approach. This is accomplished by comparing the genotypes and the phenotypes of the cells at the benign and malignant stages. The experimental approach that we have chosen for our studies is the exogenous approach. This involves introducing candidate genes into benign papillomas cells and looking for the malignant phenotype.

Previous studies using exogenous approaches have shown that members of the metalloproteases family, stromelysin and matrilysin, can cause the cells to become more invasive (Matrisian et al., 1991; Powell et al., 1993). These studies have helped to establish a causative role for metalloproteases in tumor cell invasion.

The exogenous approach has also been used to study the effects of nuclear oncogenes in tumor progression. Yuspa's laboratory has developed a benign papilloma cell line from in vivo initiated mouse skin, 308 (Strickland et al., 1988). These keratinocytes contain an activated c-ras\(^{Ha}\) oncogene and they do not produce tumors upon subcutaneously injection into athymic nude mice (Strickland et al., 1988).

Yuspa's laboratory has found that v-fos transfection can
cause the malignant conversion of the 308 cells (Greenhalgh et al., 1988). The same study also showed that two other oncogenes that code for proteins in the nucleus, E1A and myc, did not compliment the activated c-ras\textsuperscript{Ha} oncogene in causing malignant conversion. In addition, unpublished data from collaborative work between L. M. Matrisian, G. T. Bowden and S. H. Yuspa's laboratories have shown that v-fos transfection of the 308 cell also led to an increase in the level of expression for the secreted metalloprotease, stromelysin. The increased expression level of stromelysin may contributed to the invasive phenotype of these cells.

The candidate complementing gene that we have chosen to investigate is the v-jun oncogene. Jun is the transforming oncogene of the avian sarcoma virus 17 (ASV 17) that induces fibrosarcomas in infected chickens (Maki et al., 1987). The first indication that the v-jun oncogene product is a transcription factor comes from a computer search of its sequence which showed a significant degree of homology with the yeast transcription activator protein GCN4 (Vogt et al., 1987). This suggested that jun is involved in the pathways for relaying extracellular stimuli to the control of gene transcription. Further characterization of this oncogene revealed that the jun protein binds to the same DNA sequence as the human transcription activator AP-1, and antibodies raised against jun strongly cross-reacted with AP-1 (Bohmann
et al., 1987). It was later demonstrated that AP-1 is composed of the jun family of proteins dimerizing with the product of c-fos proto-oncogene (Franza, 1988; Vogt et al. 1990).

The consensus jun/AP-1 binding sequence TGAG/CTCA has been shown to be the DNA sequence that mediates the phorbol ester tumor promoter, TPA, response and is also known as the TPA responsive element (TRE) (Angel et al., 1987; Lee et al., 1987). Thus, jun is responsible for increasing gene transcription as the direct result of the cells being stimulated by TPA.

V-jun is 15-to 25-fold more potent than its cellular counterpart, c-jun, in its ability to transform chicken embryo fibroblasts in cell culture (Bos et al., 1990). In vitro transcription assays have also shown that v-jun has greater transcriptional activation activity than c-jun (Bohmann et al., 1989). However, this ability to activate transcription is specific for a given cell-line. In F9 embryocarcinoma cells, expression of v-jun stimulated the activity of both the polyoma-virus enhancer and a multimer containing the PEA1 binding site, but v-jun expression led to no detectable transcription activation in MPC11BU4 myeloma and NIH3T3 fibroblasts (Imler et al., 1988). In LMTK-fibroblasts, both c-jun and v-jun repressed the polyoma-virus enhancer activity (Schneikert et al., 1991).
Transgenic mice that contain v-jun driven by the promoter of the widely expressed H-2Kk major histocompatibility gene do not spontaneously develop tumors at an increased frequency (Schuh et al., 1990). These mice do exhibit abnormal wound repair, and following full-thickness wounding, they developed dermal fibrosarcomas (Schuh et al., 1990). The fact that only fibrosarcomas and no epidermal tumors were observed suggests that the transforming activity of v-jun may also be specific for a given cell-type.

Statement of Problem

Our original hypothesis was that the progression of tumor cells from benign to malignant stage involves a change in the genotypes of the tumor cells. Specifically, we wanted to test the hypothesis that introduction of an activated oncogene, v-jun, into a benign papilloma cell line 308, which contains the activated c-ras\(^{Ha}\) oncogene, can turn on a set of cellular genes that may play a role in tumor cell progression, including tumor cell invasion.

This hypothesis is based on the finding that jun is the nuclear transcription activator protein that mediated the cellular response for the classical phorbol ester tumor promoter, TPA. We propose that v-jun homodimers or v-jun-fos heterodimers could bind to the TPA responsive element and
cause a constitutive turn-on of TPA responsive genes. Such an event could potentially turn TPA's transient induction of a invasive phenotype of 308 cells into a permanent effect. This could then lead to the malignant conversion of these benign tumor cells.

However, the results that we obtained indicated that v-jun did not cause these benign papilloma cells to become malignant carcinoma cells, and it did not make the 308 cells more invasive. Instead, the data we have obtained showed that v-jun blocked the TPA induced invasion of the 308 cells (Tsang et al. in press). This suppressor activity was distinct from the proposed mechanisms for other known tumor suppressor genes in that it appeared to have no effect on the growth of the cells.

Our further characterization of the mechanism of this suppression indicated that v-jun transfection inhibited the TPA induction of the stromelysin gene expression. Surprisingly, TPA induction of other cellular genes were not effected. The results therefore suggested that v-jun can specifically block the TPA induction of the stromelysin gene. This may in part explain v-jun's suppression of the TPA induction of tumor cell invasion.

During the course of my dissertation project, I have developed a set of cassette vectors that can significantly improve on the efficiency of adapting DNA restriction
fragments so that it can be cloned into another restriction site (Tsang et al., 1991). This solved a common technical problem faced by researchers in molecular biology.

I have also formulated a new model with regards to the potential mechanisms of function for the 70kDa family of heat-shock proteins (hsp 70) based on the outside proposal that I have prepared for my Ph. D. preliminary exam (Tsang, 1993). This model proposed that hsp 70s can serve as a cross-linker between cellular proteins and the actin microfilaments and it may have significant implications on our current views on both hsp 70s and the cytoskeleton.
Material and Methods

Cell culture 308 cells were generously provided by Dr. Greenhalgh and Dr. Yuspa. These cells have an activated c-ras\textsuperscript{Ha} gene and produce papillomas upon transplantation and no tumors upon subcutaneous injection (Strickland et al., 1988). They were maintained in minimal essential medium (MEM) plus 8% fetal calf serum.

Plasmid DNAs pG-5-5-1, which contains v-jun was kindly provided by Dr. Peter Vogt. The 3.38 kb Bgl I/ Xba I fragment was ligated with the 1.32 kb Bgl I/ Xba I fragment from pGEM 3 to create a plasmid containing v-jun flanked by EcoR I sites.

The entire 1.8 kb gag-jun fusion gene from ASV 17 was then cloned into the EcoR I site of the mammalian expression vector, pkCR3 (kind gift from Dr. R. Breathnach). The resulting v-jun expression vector, pGJ-1 (Fig. 2), contained the gag-jun sequence driven by the SV40 promoter and also contained a SV40 poly-adenylation signal. The plasmid pSV2neo contains the neomycin phosphoribosyltransferase gene linked to the SV40 promoter (Southern et al., 1982).

Transfection 17 ug of pGJ-1 or pKCR3 were co-transfected with 3 ug of pSV2neo into the 308 cells, using the Ca PO\textsubscript{4} procedure (Graham et al., 1973). Neo-resistant clones were selected with 400 micrograms per ml of active G418 and
Southern and Northern Blots

Isolation and Southern analysis of DNA from cell lines were performed essentially as described elsewhere (Maniatis et al., 1982). 20 ug of genomic DNA were cut with EcoRI, separated on an 1% agarose gel and transferred to Genescreen blot. The DNA blot was probed with a 32P labeled v-jun specific probe that contains a 300bp Xho I/Hind III fragment of the gag gene. A 900-bp segment from the mouse genomic beta-actin gene (kind gift of Dr. M.J. Getz) was used as internal control. Densitometry scanning of the autoradiogram was used to determine the relative intensity of the bands.

Total RNA was isolated by a method (Chomczynski et al., 1987) using guanidinium isothiocyanate and separated on a 1% agarose/2.2M formaldehyde gel. Equal loading was confirmed by measurement of absorbency at 260 nm and by staining with ethidium bromide. RNA was transferred and UV cross-linked to Genescreen. These blots were probed with the v-jun specific gag insert mentioned above.

For TPA time course experiments, cells treated with TPA were harvested and total RNA was isolated, transferred and hybridized with various probes as described above. A 2.2-kb EcoRI-SalI fragment of pc-fos-3, the mouse c-fos cDNA (gift from Dr. T. Curran), a 353-bp EcoRI-AvaI fragment of the mouse c-jun cDNA (Ryder et al., 1988), a 1.8-kb PstI-EcoRV
fragment of the mouse PA-urokinase cDNA (Rorth et al., 1990) and a 1.6-kb EcoRI-EcoRI insert of the mouse stromelysin cDNA pTR11A (Matrisian et al., 1990) were used as probes for the c-fos, c-jun, PA-urokinase and the stromelysin messages.

Phorbol ester treatment For both Matrigel invasion assay and northern time course, newly confluent cells were serum starved for 12 hours prior to phorbol ester TPA-(12-O-tetradecanoyl-phobol-13-acetate) treatment. 100ng of TPA dissolved in acetone were added per ml of medium.

Matrigel invasion assay The Membrane Invasion Culture System (MICS) has been previously described (Hendrix et al., 1987). Briefly, 200,000 cells were seeded on the Matrigel-coated polycarbonate filter and incubated in the presence or the absence of TPA for 72 hours. Each chamber contained 3 ml of DMEM with 10% NU serum (Collaborative Research). The cells that invaded the Matrigel-coated filter were collected, stained and counted with the aid of a Zeiss microscope.

Statistical Analysis Data from Matrigel invasion assay were analyzed as follows: Each chamber of 12 wells contained three replicates of two cell lines with and without TPA (3x2x2=12). Within each chamber, the logarithm of the average of the three replicates was computed, yielding a with-TPA and without-TPA measure for each cell line in the chamber (a logarithmic transformation was required in order to give approximately constant variance across cell lines). The
difference between the with- and without-TPA measures were analyzed using regression methods (Draper et al., 1981) to compare the level of invasiveness across cell lines.
**VIRAL JUN ONCOGENE SERVES AS A SUPPRESSER OF PHORBOL ESTER TPA INDUCED TUMOR CELL INVASION AND STROMELYSIN GENE EXPRESSION**

**Introduction**

*Jun* is the transforming oncogene of the avian sarcoma virus 17 (ASV 17) that causes fibrosarcomas in infected chickens. The *jun* family of proteins can dimerize with the product of *c-fos* proto-oncogene to form the transcription activator complex AP-1 (Vogt, 1990). The consensus AP-1 binding sequence TGAG/CTCA has been shown to be the phorbol ester tumor promoter, TPA, responsive element (TRE) (Angel, 1987; Lee, 1987). Thus, *jun* is involved in the pathways for relaying extracellular stimuli to the control of gene transcription.

*V-jun* can activate transcription in *in vitro* transcription activation assays (Bohmann, 1989; Bos, 1990), but this activity is dependent on the cell-line (Imler, 1988). In some cell-lines such as the LMTK- fibroblasts, *v-jun* can repress the polyoma-virus enhancer activity (Schneikert, 1991). Transgenic mice that contain *v-jun* exhibit abnormal wound repair, and they developed dermal fibrosarcomas following full-thickness wounding (Schuh, 1990). No carcinomas were reported. These results also supported the
notion that the transforming activity of \textit{v-jun} may also be specific for a given cell-type.

In this study, we have investigated the transforming activity of the \textit{v-jun} oncogene using a murine epithelial keratinocyte model system (Strickland, 1988). Specifically, we addressed the question of whether \textit{v-jun} can complement activated \textit{c-ras}^{Ha} oncogene in the mouse papilloma cell-line, 308, and cause its malignant conversion. 308 cells were derived from \textit{in vivo} initiated mouse skin, and they contain an activated \textit{c-ras}^{Ha} gene with a mutation within the 61st codon (Strickland, 1988).

Our initial hypothesis was that \textit{v-jun} transfection may cause the constitutive activation of the TPA-responsive genes. Some of these genes, such as stromelysin, are thought to play a functional role in the process of malignant tumor cell invasion (Ostrowski, 1988; Matrisian, 1990). However, our results indicate that \textit{v-jun} does not cause the malignant conversion of the 308 cells. Instead, \textit{v-jun} transfection completely abolished the induction by TPA of invasion of reconstituted basement membrane matrix (Matrigel) \textit{in vitro}. In addition, our results also indicate that \textit{v-jun} can specifically suppress the TPA induction of stromelysin gene expression. This suggests a possible mechanism for inhibition of tumor cell invasion.
Results

Expression of v-jun in transfected cells

DNA and RNA were isolated from the G418-resistant lines to determine the integration and expression of v-jun gene. For Southern analysis, a 1.8 kb band was detected in four of the lines transfected with pGJ-1 (lane 3, 4, 5, 6) while the parental 308 cells (lane 1) and control cells transfected with pKCR3 vector alone (lane 2) showed no hybridization to the v-jun specific probe (Fig. 3).

A beta-actin intron probe was used as internal control to determine the relative number of copies integrated. It was shown that line vj-1 had 6x, and vj-15 had 28x the number of copies of v-jun gene integrated compared to vj-3.

Northern analysis of the same cell lines showed that the v-jun message was expressed in the same cell lines where v-jun was integrated. The levels of expression correlated with relative number of copies integrated (Fig.4).

Fluorescence activated cell sorter analysis using an antibody, PEP 1 (Oncogene Science), that recognized both c-jun and v-jun also suggested that there was more total jun protein in the vj-1 and vj-15 cells as compared to both the parental 308 and mock transfected cells (data not shown).

These results showed that line vj-1 and vj-15 have the highest levels of v-jun expression, and therefore they were
selected for further analysis.

**Tumorigenicity of the 308 cells expressing v-jun**

Vj-1, vj-15, mock transfected cells and the parental 308 cells were subcutaneously injected into nude mice to assay for malignant progression. Injection sites were palpated for tumor growth for 10 weeks. The methods were essentially the same as described elsewhere (Strickland, 1988).

In two experiments, one using $5 \times 10^5$ and another using $5 \times 10^6$ cells per injection site, there were no evidence of malignant progression for any of the cell lines.

**Matrigel invasion assay**

308 parental cells, mock transfected cells and v-jun transfected cell-lines vj-1 and vj-15 were tested for their invasive ability using the *in vitro* Matrigel cell invasion assay (Hendrix, 1987). Each cell-line was either untreated or treated with 100ng per ml of TPA.

The raw data are presented in Fig. 5 and the results are summarized in Table 1. We performed two independent sets of experiments. Experiment # 1 compared 308 with vj-15, and experiment # 2 compared 308 with vj-1 and also compared 308 with mock transfected cells.

The results from both sets of experiments indicated that all four cell lines used were essentially non-invasive
without TPA treatment (less than 0.1% of the cells seeded invaded). Upon TPA treatment, both 308 and mock transfected cells showed a significant increase in invasion while both of the v-jun transfected cell lines did not.

Experiment #1 showed that TPA treatment increased the invasion of 308 cells an average of 72.88 fold while TPA treated vj-15 cells only showed an average increase of 1.68 fold in invasion. Experiment #2 results showed that treatment with TPA increased the invasion of 308 cells an average of 10.38 fold and the mock transfected cells an average of 9.22 fold. In contrast, vj-1 cells only showed an average increase of 1.04 fold.

Statistical analysis of the data indicated that the differences between TPA induced invasion in the parental 308 cells, vj-1 and vj-15 were highly significant. Statistical analysis also indicated that the differences between 308 cells and the mock transfected cells were not significant.

The differences observed in the levels of TPA induction between 308 cells in experiment #1 and experiment #2 are statistically significant. However, due to the fact that variations often exist in thickness of Matrigel coating for each filter and also between different lots of Matrigel used, data are generally only compared within each experiment.

TPA is a potent tumor promoter and it has been shown to transiently induce a malignant phenotype (Verma, 1980). Our
results indicate that TPA treatment can significantly increase the Matrigel invasion of benign tumor cell line 308.

The abolishment of TPA induced tumor cell invasion in both v-jun transfected cell-lines was an unexpected result. This suggests that v-jun may have an inhibitory effect on TPA induced cellular invasion in the 308 cell-line.

**Phorbol ester induction of cellular genes** To further investigate the mechanism of this suppression, we studied the effects of TPA treatment on the messenger RNA levels of various TPA inducible genes. Some of these genes might play a role in tumor cell invasion.

Total RNA was isolated at various time points after TPA treatment and the RNA was probed for the induction of c-fos, C-jun, mouse PA-urokinase and mouse stromelysin (transin) messages (Fig. 6-9).

In Fig. 6 it is shown that in both vj-1 and vj-15, the TPA induction of c-fos messenger RNA was similar to that of the parental 308 cells. There was an early induction of c-fos message peaking at about 1 hr after TPA treatment. We also looked at the TPA induction of c-jun (Fig. 7) and plasminogen activator type urokinase (Fig. 8), and these genes also showed no significant difference between v-jun expressing and non-expressing 308 cells in their TPA induction of message. However, the TPA induction of stromelysin messenger RNA in
both vj-1 and vj-15 cells was markedly different than that for the parental 308 cells (Fig. 9). 308 cells normally have some detectable low level of stromelysin message. After TPA treatment, there was a significant induction peaking at about 12 h after treatment.

In both vj-1 and vj-15 there was no detectable message at 0 h. After TPA treatment, vj-1 showed a much lower level of induction than 308 and the induction peaked at about 3 h after TPA treatment. In vj-15, where v-jun expression level was highest, there was no detectable TPA induction of stromelysin gene expression.
Fig. 2 **pGJ-1 expression vector** The 1.8 kb fragment of the gag-jun gene was cloned into the EcoRI site of the mammalian expression vector, pKCR3. The gag-jun gene is under the control of the SV40 promoter and SV40 polyadenylation sites.
Fig. 3 Southern blot analysis of the v-jun transfected cell lines. DNA was digested with EcoR I and analyzed as described in "Material and Methods." The blot was probed with a $^{32}$P labeled v-jun specific probe that contains a 300bp Xho I/Hind III fragment of the gag gene and a 900-bp segment from the mouse genomic beta-actin gene. The numbers indicated the relative levels of v-jun after correcting for the beta-actin internal control.
Fig. 4 Northern blot analysis of the v-jun transfected cell lines. Total RNA was analyzed as described in "Material and Methods." The blot was probed with a $^{32}$P labeled V-jun specific probe that contains a 300bp Xho I/Hind III fragment of the gag gene.
Fig. 5 TPA induction of Matrigel invasion of the \textit{v-jun} transfected cell lines. Each of the numbers (1-8) on the graph represented the averaged results from an independent Matrigel invasion chamber plotted on a log scale. For all eight chambers, a triplicate of the samples wells were assayed for each treatment group. 200,000 cells were seeded per sample well, and after 72 hours, the cells invaded through the Matrigel were collected and counted using a Zeiss microscope.
Table 1: Results of Statistical Analysis

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<th>Cell Line</th>
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<tr>
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<tr>
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<td>1.68</td>
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<tr>
<td>Line 308</td>
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<td>(5.00,21.54)</td>
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<tr>
<td>Mock</td>
<td>9.22</td>
<td>(3.28,25.91)</td>
<td>0.82 (^{(5)})</td>
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\(^{(1)}\) Fitted estimate (resulting from regression analysis) of the ratio between the number of cells invading with TPA to the number of cells invading without TPA (i.e. "fold increase").

\(^{(2)}\) P-value for comparison of the invasiveness of VJ-15 to 308.

\(^{(3)}\) P-value for comparison of the invasiveness of 308 in experiment #1 to 308 in experiment #2.

\(^{(4)}\) P-value for comparison of the invasiveness of VJ-1 to 308

\(^{(5)}\) P-value for comparison of the invasiveness of Mock to 308.
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Fig. 6 Northern analysis of c-fos gene expression after TPA treatment. Cells were serum starved for 12 h prior to TPA treatment. Total RNA was isolated from cells at each time point. Northern analysis was performed as described in the "Material and Methods". A 2.2-kb EcoRI-SalI fragment of pc-fos-3, the mouse c-fos cDNA was used as the probe for the c-fos message.
Fig. 7 Northern analysis of c-jun gene expression after TPA treatment. Cells were serum starved for 12 h prior to TPA treatment. Total RNA was isolated from cells at each time point. Northern analysis was performed as described in the "Material and Methods". A 353-bp EcoRI-AvaI fragment of the mouse c-jun cDNA was used as the probe for the c-jun message.
Fig. 8 Northern analysis of PA-Urokinase gene expression after TPA treatment. Cells were serum starved for 12 h prior to TPA treatment. Total RNA was isolated from cells at each time point. Northern analysis was performed as described in the "Material and Methods". A 1.8-kb PstI-EcoRV fragment of the mouse PA-urokinase cDNA was used as the probe for the PA-urokinase message.
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Fig. 9 Northern analysis of stromelysin gene expression after TPA treatment. Cells were serum starved for 12 h prior to TPA treatment. Total RNA was isolated from cells at each time point. Northern analysis was performed as described in the "Material and Methods". A 1.6-kb EcoRI-EcoRI insert of the mouse stromelysin cDNA pTR11A (Matrisian, 1990) was used as the probe for stromelysin message.
Discussion

In contrast to a previous report that transfection of 308 cells with an activated fos oncogene, \( v\text{-}fos \), led to their malignant conversion (Greenhalgh, 1988), our data show that the activated jun oncogene, \( v\text{-}jun \), is not able to compliment the activated \( c\text{-}ras^{H\text{a}} \) oncogene and cause the malignant conversion of the 308 cells. Instead, our findings indicated that \( v\text{-}jun \) may function as a suppresser of TPA induced tumor cell invasion and stromelysin gene expression. This suppression appears be specific for the process of invasion since neither the growth rate nor the morphology of 308 cells transfected with \( v\text{-}jun \) were significantly altered.

Both \( jun \) and \( fos \) are major components of the AP-1 transcription activation complex that has been shown to mediate the induction of cellular genes by the phorbol ester tumor promoter TPA (Vogt, 1990; Angel, 1987; Lee, 1987). A potential explanation for the observed differences between \( v\text{-}jun \) and \( v\text{-}fos \) in complimenting the activated \( c\text{-}ras^{H\text{a}} \) oncogene may lie in the differences in the steady-state expression levels of their cellular counterparts.

In 308 cells, the steady-state levels of \( c\text{-}jun \) were relatively high and the expression level increased only slightly after TPA treatment (Fig. 7) (Holladay, 1992). The steady-state level of \( c\text{-}fos \) gene expression in 308 cells was
below the level of detection (Fig. 6), and this level was dramatically increased after TPA treatment. Thus, it is possible that constitutive overexpression of \textit{v-fos} may cause significant changes in AP-1 regulation of genes in 308 cells, while the high steady-state level of \textit{jun} expression in 308 cells would suggest that \textit{jun} is not a limiting factor. Therefore, expression of the \textit{v-jun} oncogene would have little effect in changing the AP-1 regulation of most AP-1 regulated cellular genes.

Unpublished data from collaborative work between L. M. Matrisian, G. T. Bowden and S. Yuspa supports this idea. These workers have shown that the malignantly converted \textit{v-fos} transfected 308 cells have an increased steady-state level of message for stromelysin which is one of the genes known to be regulated by AP-1.

However, our results in Fig. 9 indicated that neither of the \textit{v-jun} transfected cell lines \textit{v-j 1} and \textit{v-j 15} expressed detectable steady-state message for stromelysin. Our data indicating that \textit{v-jun} transfection specifically suppressed the TPA induction of the stromelysin gene expression was an unexpected result. Since TPA induction of other AP-1 regulated genes appeared to be normal in both \textit{vj-1} and \textit{vj-15} cells, it is unlikely that there is a defect in the signal transduction pathway for TPA induction in these two cell lines.
These data would also tend to argue against either general "squelching", "quenching" or "blocking" (Brown, 1993) of the cellular factors as the mechanism for the suppression of stromelysin gene induction. We speculate that v-jun, which has altered binding specificity in vivo as compared to c-jun (Hadman, 1993), may interact with some specific regulatory elements within the stromelysin gene promoter. V-jun could potentially block stromelysin gene induction through a specific "squelching", "quenching" or "blocking" mechanism.

The most probable site in the stromelysin gene promoter for jun's interactions with the DNA is the TPA responsive element (TRE). The TRE is located at about 50bps upstream of the transcription start site in both the rat (Kerr et al., 1988) and the mouse (our lab's unpublished data) stromelysin gene promoter. There is a highly conserved arrangement of three regulatory elements near the TRE in the stromelysin gene promoter. The TRE and the ras-responsive element (RRE) (Owen et al., 1990) formed a 6bp palindrome sequence flanking the 9bp TGF-beta inhibitory element (TIE) (Kerr et al. 1990) (Fig. 10). This remarkable arrangement of the three elements suggested that these three elements may interact in a synergistic and/or competitive manner. The 9bp distance is significant in that it represents one complete turn of DNA double helix. This gives the TRE-TIE-RRE sequence a potential
to form DNA secondary structures that may be important for regulation of the stromelysin gene.

It can also not be ruled out that v-jun has caused the constitutive expression of an unknown suppressor gene which is in turn responsible for blocking stromelysin gene induction. A similar indirect mechanism of suppression was proposed for c-jun and v-jun's repression of the polyoma-virus enhancer (Schneikert, 1991).

We have observed a correlation between the suppression of TPA induced tumor cell invasion and the suppression of TPA induced stromelysin expression. The metalloproteinase stromelysin has been implicated as playing a functional role in tumor cell invasion. It has been shown that the messenger RNA of mouse stromelysin is expressed more abundantly in malignant than in benign tumors (Matrisian, 1986), and that increased steady-state expression of the stromelysin messenger RNA was correlated with malignant tumors with the greatest probability to metastasize (Ostrowski, 1988).

In addition, Matrisian and co-workers have demonstrated that expression of activated stromelysin in papilloma-derived cells was sufficient to increase their invasion in the Matrigel invasion assay (Matrisian, 1991). Although we have not directly shown that v-jun's suppression of TPA induced invasion is caused by v-jun's suppression of TPA induction of stromelysin gene expression, our data are consistent with the
idea that matrix metalloproteinases and specifically, stromelysin plays a functional role in the process of tumor cell invasion.

The results presented here provided further supporting evidence for previous conclusions from other laboratories that v-jun's transforming and the transactivation ability were cell type specific. One of the major differences between the pathway to full malignancy between fibroblasts and epithelial cells is that epithelial tumors must invade through the basal lamina (also known as the basement membrane). This may involve the expression of secreted metalloproteinases such as stromelysin.

Knowledge of how v-jun can specifically suppress the process of epithelial tumor cell invasion may eventually lead to therapeutic strategies targeting the invasion step (Liotta et al. 1991). Such therapy strategies could potentially have less toxic side effects than cancer therapies that suppressed the growth of the tumor cells.
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Fig. 10 The conserved TRE-TIE-RRE region in the rat and mouse stromelysin gene. The TPA responsive element (TRE) forms a 6bp palindrome with the ras-responsive element (RRE) sequence (underlined). The 9bp sequence in between TRE and RRE is the TGF-beta inhibitory element (TIE).
A Set of Cassette Cloning Vectors for Rapid and Versatile Adaptation of Restriction Fragments

A set of two cassette cloning vectors, p34E and p34H, were created to allow for rapid and versatile adaptation of DNA restriction fragments for cloning into another restriction site. Both vectors were constructed by ligating one fragment derived from the pGEM3 (Promega, Madison, WI) plasmid with one fragment derived from the pGEM4 (promega) plasmid, each containing a portion of the ampicillin resistance marker and all of the multiple cloning sites (MCS). The p34E plasmid contains two inverted repeated (IR) copies of the MCS flanking a single EcoRI site (Figure 10). The p34H plasmid also contains two IR copies of the MCS, but they flank the single HindIII site (Figure 11). Restriction fragments of any of the 13 sites presented in the MCS can be easily adapted for any of the other 12 remaining sites by first cloning the fragment of interest into the cassette vectors and then cutting the piece back out using the double-flanking site of choice. The DNA fragment can also be adapted to contain two different flanking sites for directional cloning. This is accomplished by first digesting the inner restriction sites. This will yield DNA fragments of both sense and anti-sense orientations that can be used for directional cloning.
This set of cassette cloning vectors can be used as general purpose plasmids for cloning and in vitro transcription. They have, however, proven to be especially valuable when cloning DNA into large expression vectors with only few available cloning sites, such as the defective retroviral vector pLJ (Korman, 1987) and the mammalian expression vector pHB Apr-1-neo (Gunning, 1987). p34E has been used successfully in adapting EcoRI fragments for both SalI and BamHI cloning sites of the pLJ vector and for directionally cloning EcoRI fragments into the HindIII and BamHI sites of the pHB Apr-1-neo vector while eliminating the need for such tedious steps as blunt-end ligation and the use of adapters.

While trying to work out the techniques involved in cloning, I have constructed a set of cassette cloning vector, p34E and p34H, that has greatly increased the efficiency of cloning a restriction fragment into another site. Restriction fragments produced by cutting with any of the eleven restriction enzymes can be adapted for any of the eleven other sites by simply ligating the fragment into either p34E or p34H and cut back out with the enzyme of choice. This set of vectors have been very useful in both my experiments and those of other people in our lab, particularly for cloning genes into large expression vectors where there are generally a limited number of sites available.
These vectors were published in the March 1991 issue of Biotechniques, and we have since received over 170 requests from countries around the world for them.
Fig. 11 **Construction of the p34E plasmid** Both pGem 3 and pGem 4 (Promega) were digested with EcoRI and BglI. The pieces containing all of the multiple cloning site and part of the ampicillin resistance gene (striped) were isolated and ligated together to yield the p34E vector.
Fig. 12 **Construction of the p34H plasmid** Both pGem 3 and pGem 4 (Promega) were digested with HindIII and BglI. The pieces containing all of the multiple cloning site and part of the ampicillin resistance gene (striped) were isolated and ligated together to yield the p34H vector.
New model for 70 kDa heat-shock proteins' potential mechanisms of function

INTRODUCTION

The intracellular environment is extremely viscous. Its texture more resembled a gelatinous substance than a perfect solution. Because diffusion may be a prohibitively inefficient way for transporting macromolecules, a highly developed active transport system would be necessary as the cells evolved toward greater size and complexity.

CYTOSKELETAL MATRIX CROSS-LINKERS

The cytoskeletal matrix, also known as the cytoskeleton, is composed of an intricate network of filaments, motor enzymes and associated proteins. It is the basic framework for cellular organization, and it also supports the cellular transport apparatus. Although the cytoskeletal matrix has been implicated in many essential cellular processes, a significant gap in our current knowledge is what physically linked the cytoskeletal matrix to all the cellular components.

The simplest model for coupling cellular proteins to the cytoskeletal matrix is for them to bind to the matrix
directly (Tsang, 1993). Such a model is dependent upon the discovery of a set of cytoskeletal matrix cross-linker molecules. These hypothetical cross-linker molecules must have at least two binding sites, one to bind the cytoskeletal matrix, and another to bind various cellular proteins.

The most probable sites on cellular proteins to interact with cross-linkers are the targeting sequences. Cellular targeting sequences are generally consisted of short stretches of charged amino acids. They are necessary and often sufficient for mediating transport of proteins to a particular cellular compartment. The fact that targeting sequences can be sufficient for mediating protein transport suggests that they must interact with the transport apparatus at some time.

MODEL FOR HSP 70S AS CROSS-LINKERS

A number of recent discoveries have suggested that the 70 kDa family of heat-shock proteins (hsp 70s and hsc 70s) served as cross-linker molecules between transported cellular proteins and the actin microfilaments. To establish hsp 70s as cytoskeletal cross-linkers, it will be necessary to demonstrate that hsp 70s have two binding sites, one for those proteins targeted for transport, and another one for actin.
The structure of hsp 70s contains two major domains (Gething and Sambrook, 1992). The sequences at the C-terminus are not very conserved among the different family members of the hsp 70s. This region can bind with an enormous variety of both folded and unfolded cellular proteins. It is also considered to be the substrate recognition domain that interacted with cellular proteins targeted for transport.

Hsp 70 proteins are involved in facilitating protein transport to several different organelles. This included the nucleus, the endoplasmic reticulum, mitochondria and lysosomes (Gething and Sambrook, 1992; Golfarb, 1992; Deshaies, 1988; Chirico, 1988; Shi 1992; Chiang, 1989; Dingwall, 1992; Imamoto, 1990; Imamoto, 1993). In those cases where the sites of interaction have been characterized, the mechanism appears to involve direct binding of hsp 70 proteins with cellular targeting sequences (Chiang, 1989; Dingwall, 1992; Imamoto, 1990; Imamoto, 1993).

Dice and co-workers have identified a protein that specifically recognized the lysosomal targeting sequences (KFERQ and related sequences). This protein was subsequently shown to be a member of the hsp 70 family of proteins, hsc 73 (Chiang, 1989).

A similar mechanism was discovered for hsp 70s' role in facilitating protein transport to the nucleus. Yoneda and co-workers have isolated a receptor protein that specifically
binds to the positively charged nuclear location signal sequences (NLSs) (Imamoto, 1990). Based on its molecular weight, isoelectric point, cellular localization, and partial amino acid sequencing, this NLSs receptor protein has now been identified to be hsc 70 (Imamoto, 1993).

This finding is particularly relevant for the studies of hsp 70s' possible mechanisms of function since evidence have shown that protein transport to the nucleus is independent of their conformational states. Current data therefore supported that hsp 70s' C-terminal substrate recognition domain bind to the protein targeting sequences and direct protein transport to two organelles, the nucleus and the lysosomes.

The 44K, N-terminal domain of hsp 70s contains the ATPase activity. Its sequence is highly conserved among all the members of the hsp 70s family (Gething and Sambrook, 1992). The three-dimensional structure of this fragment has been determined by X-ray crystallography (Flaherty, 1990).

It revealed that this part of hsp 70 has nearly the same structure as the actin monomers that polymerize to form the actin microfilament network (Flaherty, 1991). These data would support the notion that the 44K ATPase portion of hsp 70s and actin may share similar binding sites and that they may bind to each other.

Studies using a monoclonal antibody against hsp 70 have revealed that the hsp 70 proteins have an identical pattern
of distribution as the actin microfilaments (La Thangue, 1984). This suggests that hsp 70s is an actin-associated proteins.

The most convincing set of evidence for hsp 70s' association with actin was found by Margulis and Welsh (Margulis, 1991). They have discovered that hsp 70 binds to actin directly. The data indicated that the tight association between actin and hsp 70s is constitutive, and that it is due to hydrophobic types of interactions.

These results are consistent with earlier evidence obtained by Morimoto's lab that showed a protein with same molecular weight as actin, is the major hsp 70-binding protein throughout the cell cycle and after heat shock (Milarski, 1989).

Researchers studying actin-binding proteins are also beginning to find that hsp-70 binds to actin. Condeelis and co-workers have isolated a 70 kDa actin-binding protein called aginactin (Sauterer, 1991). Recently, at the thirty-second annual meeting of the American Society for Cell Biology (ASCB), it was reported that cloning and sequencing of aginactin cDNA showed that aginactin is hsp 70 (Eddy, 1992).

Thus, data from different labs using independent experimental approaches have converged to the same conclusion that hsp 70 binds to actin. The natures of these bindings are
distinct from hsp 70s' association with other cellular proteins in that different binding sites appeared to be involved.

These results showed that a significant body of experimental evidence has accumulated in the literature to justify proposing a new model for hsp 70s' function. The cross-linker model proposes that hsp 70 binds to cellular proteins through its C-terminal substrate recognition domain. The binding sites on the proteins include the protein targeting sequences, but other binding sites are certainly possible.

In eukaryotic cytosol, hsp 70 is proposed to bind with actin through its highly conserved N-terminal domain. The nature of interaction appears to be hydrophobic, and it is regulated by hydrolysis of ATP (Fig. 12).

APPLICATION OF THE CROSS-LINKER MODEL

Hsp 70 family of proteins binds to a variety of proteins while effecting the folding, assembly, disassembly and transport of these cellular proteins and structures (Gething and Sambrook, 1992; Lindquist, 1988; Craig, 1991).

Although this model is derived by analyzing hsp 70s' involvement with the cytoskeletal transport apparatus, it can be used to interpret other known hsp 70s' functions. The
roles of hsp 70s and the cytoskeletal matrix in these diverse and seemingly unrelated processes can all potentially be more precisely defined by the cross-linker model.

One of the best established roles of the cytoskeletal matrix is to provide solid support for various cellular components. This could play a significant role in the process of protein folding. Our current understanding of protein folding is based on the studies of Christian Anfinsen (Anfinsen, 1973), who was awarded the Nobel Prize for his work. These classical studies clearly showed that protein folding is a process governed by the free energy states of the molecules, and therefore firmly established the elegant symmetry between protein structures and the genetic code.

Beckmann et al. have determined that hsp 70 binds to nascent polypeptide chains as they emerged from the ribosomes (Beckmann, 1990). The cytoskeletal matrix can serve to physically separate the immature polypeptides so that they do not interact with each other before the folding process is completed, thus preventing protein agglutination. This may explain some of the observed differences between protein folding in vitro and in vivo without having to modifying the principle of self-assembly in protein folding processes.

The cytoskeletal matrix may also increase the efficiency of the process of folding. Coupling the newly synthesized protein to the cytoskeletal matrix would restrict the
Brownian motions of a long chain polypeptide and provide a consistent starting point for folding to begin. This may stabilize the formation of weak hydrogen bonds between distant amino acids and lower the free energy barriers in these processes.

The efficiency of the assembly process can potentially be greatly improved through attachment with a solid support. Binding to the matrix eliminates the random rotations and the thermal vibrations of the molecules. If both the positions and the orientations of one or more components are fixed in such a way that the accessibility of the binding sites is optimized, the rates of interactions may increase significantly. A similar analogy has long been used to explain the mechanisms of action for enzyme catalysis.

The cytoskeletal transport apparatus has to bring the individual components together before the assembly process can begin. The final assembled products have to be delivered to its proper cellular compartments. Once these cellular components reach the organelles, they are transported across the membranes by an unknown transport apparatus.

Although the actin microfilaments have been known to bind directly with cellular membranes (Luna, 1991), it is not clear if the membrane transport apparatus is associated with the cytoskeletal matrix. In any event, the cross-linker molecules may be required for connecting cellular proteins to
the membrane transport apparatus.

Physical attachment to the cytoskeletal matrix can couple various cellular components to the mechanical forces generated by the motor enzymes. This is particularly relevant in the processes of disassembly where it often requires destabilization of existing intra- and inter-molecular bonds. The mechanical energy generated by the cytoskeletal matrix based motor enzymes could be directly applied onto these bonds if some or all of the components involved were coupled to the matrix through the cross-linkers.

The role of hsp 70s outside of the eukaryotic cytosol is not at all clear. There is some evidence suggesting that a filamentous matrix-like structure is present in the prokaryotic cells (Meng, 1980; Gobel, 1981; Stevens, 1991). Whether this structure represents the prokaryotic version of the cytoskeletal matrix is totally unknown.

In both prokaryotes and the eukaryotic organelles, membranes generally served as the supporting structures for various cellular components. Attachment to these membranes would have many of the same advantages as attachment to the cytoskeletal matrix for folding and assembly.

Since the evolution of membranes must have occurred long before the development of the cytoskeletal matrix, it is conceivable that the eukaryotic cytoskeletal matrix cross-linkers have evolved from prokaryotic membrane cross-linkers.
Thus, hsp 70s family of proteins may still be involved in attaching proteins to membranes in both prokaryotes and the eukaryotic organelles.

A significant number of heat-shock proteins are associated with the cytoskeletal matrix (Wang, 1981; Reiter, 1983; Leicht, 1986; Murti, 1988). The functions of heat-shock proteins may be directly linked to the functions of the cytoskeletal matrix.

One of the most dramatic effects after heat-shock is the near instantaneous collapse of the cytoskeletal matrix (Wiegant, 1987; Glass, 1985). A major function of heat-shock proteins could be to reform the cytoskeletal matrix after it has been damaged.

If heat-shock proteins are involved in the functions of the cytoskeletal matrix, they should be induced by any event in which the cytoskeletal activities such as assembly and transport have been increased. This may explain why heat-shock proteins are induced by such an enormous variety of stimuli, some of them like the serum growth factors are not necessarily harmful to the cells.

A very important feature of the cross-linker model is that it provides an interpretation for the extraordinary conservation of the hsp 70s. The set of specific demands on the structure of the cross-linkers could maintain a great deal of selective pressure on their sequences.
Cross-linkers must be able to form at least two high affinity bonds. The strength of bonding must be strong enough to withstand the stress generated during transport of bulky components. In addition, the overall folding of the cross-linkers must be stable enough so that it will not partially denature when the stress is applied onto its structure. Such stringent functional constraints would have caused both the overall folding of the protein and its surface binding domains to remain conserved throughout evolution.

Hsp 70s' potential mechanisms of function have been one of the most actively discussed problems in biology (Pelham, 1986; Ellis, 1987; Rothman, 1989; Ellis, 1991). It is currently believed that hsp 70s served as molecular chaperones, and that they mediate the folding, assembly, disassembly and the transport of various cellular proteins by inducing them to assume the "proper conformations" for that particular cellular function.

The molecular chaperone models have been extremely helpful in stimulating and guiding the research for hsp 70s' function, including the development of the cross-linker model. However, since they were proposed prior to the information regarding to hsp 70s' structure and its binding with both the targeting sequences and the cytoskeletal matrix were available, a new model would be needed to account for these new experimental evidence.
It must be emphasized here that data that has been previously interpreted by the molecular chaperone models can also be interpreted by the cross-linker model. This is because some of the basic elements of the chaperone models are incorporated in the cross-linker model.

Since binding between hsp 70s and polypeptides occurs before translation is complete (Beckmann, 1990), proteins may not be able to achieve their mature conformations until they are released from the cross-linker molecule. Hsp 70s' binding with the nascent polypeptides would have imposed specific constraints on the evolution of productive polypeptide folding pathways.

PERSPECTIVES

The model proposed here is necessarily over-simplified in an attempt to elucidate basic principles from the overwhelming complexities of the cells. It is rather obvious that we have just barely started learning about the functions of hsp 70s, the cytoskeletal matrix and other potential cross-linker molecules.

The study of the cross-linkers is an especially rewarding field in biology because their unique nature makes it possible for us to learn more about processes at both ends of the molecule than we could ever achieve by studying either
end alone. Once the roles of these cross-linkers are determined, the pace of discoveries in many areas of biology may begin to accelerate.
Fig. 13 Proposed mechanism for hsp 70's cross-linker function: The N-terminal ATPase fragment of the hsp 70s is proposed to bind the actin microfilaments through hydrophobic interactions. The hsp 70s' C-terminal substrate recognition domain is proposed to bind cellular proteins through electrostatic interactions. The potential binding sites include protein targeting sequences.
CONCLUSIONS

The answer to the original hypothesis that I attempted to test was no. V-jun oncogene does not complement the activated c-ras$^{Ha}$ oncogene and cause the malignant conversion of the benign papilloma cells, 308. Instead, our data indicated that v-jun acted as a suppressor of the TPA induced tumor cell invasion and stromelysin gene expression.

Currently, there are a growing number of tumor suppressor genes that have been discovered (Vogelstein, 1993). Their mechanisms of action are not very well understood but they appeared to be extremely diverse.

The two best characterized suppressor genes are RB1 and p53. Both of these genes encoded gene products that are nuclear proteins and bind directly to the DNA. Re-introduction of either of these genes into tumor cells can suppress the growth of these cells (Baker, 1990; Huang, 1988).

There are also a number of genes that encode products that have been shown to suppress the process of invasion and metastasis (Liotta, 1993). TIMP-1 and TIMP-2 (tissue inhibitors of metalloproteinases) have both been shown to bind and inactivate metalloproteinases and they are both inhibitors of tumor cell invasion in vitro (Liotta, 1993).

The best characterized metastasis suppressor gene is
nm23. It encodes a NDP kinase that catalyzes the transfer of a phosphate group from triphosphate nucleotides to diphosphate nucleotides (Liotta, 1993). It is currently not clear how such a ubiquitous enzyme can act as a suppressor for metastasis.

Our finding is a unique opportunity to add to the body of knowledge regarding the potential mechanisms for tumor suppressor genes. V-jun is a well-characterized activated oncogene that has been extensively studied in fibroblast systems (Maki et al., 1987; Bos et al. 1990). It is a known transcription activator and many of its cellular interactions have been firmly established.

The fact that v-jun specifically suppressed the stromelysin gene induction suggested that there are important features in the stromelysin promoter that may provide exploitable differences for future gene therapy strategies. It should also lead to further insight into the basic mechanisms that allow transcription factors to interact with specific regulatory sequences of cellular genes.

Thus, it is with a degree of sadness as I approach the end of my graduate career. The simple question that I asked in the beginning has led to many more new ones. They are all very interesting and important questions, but they will perhaps be more interesting and important to me than they can ever become to anyone else.
A Ph. D. dissertation takes an extraordinary amount of time in a young person's life. During this time he or she gets to know more about a specialized topic than anyone else in the whole world. The risk is that by the time she or he graduated, they started to view the whole world in terms of that specialized topic.

It is for this reason that I felt especially fortunate to have a great group of teachers and an intellectually stimulating environment for my graduate education. Instead of becoming terminally differentiated, I felt that graduate school was merely my initiation and promotion stage.

My dissertation project has been marked by unexpected experimental findings and opportunity to explore new ideas about different topics. This is in part a reflection on the diversity of projects that is surrounding me.

Dr. Bowden's lab is not only involved in study of tumor progression, there are also on going projects regarding human prostate cancer, ras oncogene activation, radiation induction of tumors, cloning and mechanisms of tumor suppressor genes, cell differentiation, transcription control and cell to cell interactions.

By being constantly exposed to these different fields, I have the opportunity to gain a wide perspective on the problems of cancer. Together with the excellent courses that I have taken in the Department of Microbiology and Immunology
and the constant guidance of my mentors, I hope that I have achieved a good beginning for learning what are the important questions in science.
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