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By

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ABSTRACT

A BIOCHEMICAL AND MOLECULAR ANALYSIS OF RAF ONCOGENE TRANSFECTED RAT LIVER EPITHELIAL CELLS

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Viral raf oncogene subcloneed into a mammalian expression vector, EHneo(plasmid), was transfected into the non-tumorigenic and gap junctional intercellular communication (GJIC)-competent cell line, WB-F344, to study the effects of the expression of the oncogene on GJIC and tumorigenicity of the tranfected cells. A total of six G418-resistant clones has been isolated for analysis. One of the transfected clones exhibited spindle-shaped morphology, while the others showed the same morphology as the wild type parental cells. The morphologically transformed clone was the only one which showed expressed v-raf mRNA and raf kinase signals as examined by northern blot and western blot analyses, respectively. Among all the v-raf transfectants examined, only those clones which had v-raf mRNA expression were capable of anchorage-independent growth in soft agar.

GJIC was examined in v-raf-, pSV-neo (plasmid control) and the parental WB-F344 cells using the scrape loading-dye transfer technique. It was found that the transformed clone had reduced intercellular communication compared to the parental and plasmid control cells. Northern analysis showed that the level of connexin 43 mRNA was unchanged in the transformed cells compared to WB-F344 cells. Furthermore, the v-raf transformed cells treated with TPA (100 ug/ml) overnight showed reduced GJIC compared to the same cells without TPA treatment.

These results indicate that the expression of viral raf oncogene might induce neoplastic transformation, although the frequency of such transformations is low. The results also reveal that raf protein kinase might downregulate GJIC and affect the stability of PKC translocation via directly or indirectly pathway.

To my parents with love

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INTRODUCTION

Oncogenes are being extensively studied for the mechanism of carcinogenesis. Raf oncogenes encode proteins with protein-serine/threonine kinase activity. There is ample evidence that raf protein plays an important role in transmitting signals from cell surface receptors to the nucleus. However, both the exact mechanisms by which raf is functioning and the down stream targets of raf proteins remain to be elucidated.

One form of intercellular communication is mediated by membrane channels known as gap junctions. The junctional communication has been implicated in the regulation of tissue homeostasis, cell growth and differentiation (Vitkauskas et al. 1985). Many tumor cells showed either significant reduction or functional absence of gap junctions. The complete loss of GJIC of the tumor cells has also been correlated with their metastatic potential (Leiberman et al.1991). Furthermore, many non-genotoxic chemicals such as the phorbol ester, TPA were shown to inhibit GJIC and were termed, tumor promoters. The phobol ester tumor promoter, TPA is also a potent inhibitor of GJIC in many cell types as well as an activator of protein kinase C (PKC) , a serine/threonine kinase. The activated PKC presumably phosphorylates gap junction protein, resulting in blocking the intercellular communication (Oh et al. 1988). It was found that treatment cells with TPA, the activity of raf protein kinase increased as well as protein kinase C (Morrison et al. 1988). The similarity between PKC and raf protein is thus suspected. In the studies presented in this thesis study, the v-raf transfected cells were examined to determined whether stable expression of v-raf kinase can downregulate GJIC .

Mutational activation of the c-raf-1 gene has been observed in many hepatocellular carcinomas and neoplastic nodules (Beer et al. 1988; Ishikawa et al. 1985). Thargiersson and associates at the National Cancer Institution demonstrated that a rat liver epithelial

(RLE) cell infected with v-raf (3611-MSV) virus alone were only weakly tumorigenic and were not capable of growing in soft agar (Worland et al. 1990).

The WB-F344, rat liver epithelial cell line was isolated from the liver of an adult male Fisher-344 rat by Dr. Grisham of the Department of Pathology, University of North Carolina, Chapel Hill (Tsao et al. 1984). There are two major reasons that the WB-F344 cells were chosen for this study. First, these cells are known to perform high degree of GJIC. Second, the cell line is immortal but non-tumorigenic. The introduction of other oncogenes,i.e. activated H-ras, and Neu, have been found to transform these cells into tumorigenic cells (De Feijter et al. 1990).

In this study, the oncogenically active v-raf was transfected into the non-tumorigenic liver epithelial cell line, WB-F344 ,to determine if the expression of the v-raf protein might down regulate GJIC and induce neoplastic transformation of these cells.

LITERATURE REVIEW

Oncogenes

Oncogenes are a critical set of genes that become altered in the course of carcinogenesis (Hartmut et al. 1983). They were first described as retrovirus-encoded genes that produced tumors in birds and rodents. These genes were later shown to be dominant mutated forms of host genes (proto-oncogenes) that might have been picked up by the retroviruses (Bishop, J.M., 1983). It is generally believed that proto-oncogenes are normal cellular genes and have an essential physiological role. They have been shown to be involved in the regulation of cellular proliferation and differentiation (Rijsewijk 1987, Muller 1986, Bar-Sagi 1985 and Liotta 1991). However, it appears that the ability of many oncogenes to induce neoplastic transformation arises from inappropriate expression or function of proto-oncogenes that normally regulate cell proliferation.

Classification of Oncogenes

Oncogenes can be broadly grouped on the basis of their presumed function and predominant properties. The presently known oncogenes can be catergroized into five classes: growth factors (eg. sis), protein-tyrosine kinases (eg. src), Guanine Nucleotide binding proteins (eg. ras), nuclear oncogenes (eg. erbA), and protein-serine/threonine kinases (eg. raf). As mentioned previously, cellular oncogenes play very important roles in cellular homeostasis and growth control.

(a) Growth Factors

Cellular sis oncogene encodes a protein which is homologous to the B-chain of platelet-derived growth factor (PDGF) (Robbins 1983, Waterfield 1983). When the sis gene is activated, the B-chain of PDGF is constitutively synthesized. These cells are then stimulated to proliferate, and become independent of one or more exogenous growth

factors.

(b) Protein-tyrosine kinase

Some proto-oncogenes (eg. src, v-erbB) encode proteins that are located near to the inner surface of the cell membrane and have kinase activity(Downward 1984, Collett 1978). This membrane-associated protein kinases phosphorylate cellular proteins on tyrosine residues, thereby act as essential components of a messenger system, transmitting and applying signal from the surface to the interior of the cell.

(c) GTP-binding proteins

Ras family genes are the representatives in this group. Normal ras protein associates with the inner surface of the plasma membrane, where it interact with effector molecules to control cell division. This interaction is controlled by the conformation of the ras protein, which is either a GTP-bound active state or a GDP-bound inactive state.

In normal cells, active ras proteins are rapidly converted to inactive forms by an intrinsic guanosine triphosphatase (GTPase) activity(Hall 1990). In tumor cells, the mutant ras proteins remain in their active conformation for abnormally long periods and have an important step in the transformation process. In neurofibromatosis, the GTPase activating protein (GAP) is disabled and cannot hydrolize ras-bound GTP, resulting in prolonged ras activation, uncontrolled cell growth and tumor formation (Xu et al. 1990).

(d) Nuclear Oncogene

There are many kinds of nuclear oncogenes, such as myc, fos, myb, and jun. The proteins which are encoded by these oncogenes act as stimulator of gene expression; for example, the oncogene erbA functions as a transcription factor.

(e) Protein-serine/threonine kinase

The raf oncogene is the major candidate in this group. The roles which raf proteins play in cell growth control will be discussed below.

Activation of Oncogenes

It is now believed that cancer is a disease involving malfunctioning cellular genes. In fact, several mechanisms of activation have been identified from an analysis of DNA extracted from human tumors:

(a) Chromosomal translocation

It has been known that non-random chromosomal aberrations occur in human cancers. A good illustration is provided by studies of Burkitt's lymphoma(BL), a B-cell malignancy common in Africa. It was found that 100% of the lymphoma cells in BL exhibit a reciprocal translocation involving chromosome 8 at band 8q24. The translocation appears to affect the expression of c-myc by a number of mechanisms, the overproduction of the myc gene product via a translational mechanism may be a common feature for these cells (Siato et al. 1983). The translocation is now known to involve the c-abl oncogene on chromosome 9 and the bcr gene on chromosome 22 resulting in a bcr-abl protein which posses phosphotyrosine kinase activity (Heisterkamp 1983).

(b) Gene mutation

Mutation within the coding sequence of an oncogene is an important mechanism of activation, resulting in the production of aberrant protein. Such mutations can be induced by exogenous mutagens such as benzopyrene (carcinogen in tobacco) in lung cancer (Takahashi et al. 1991), aflatoxin in hepatocellular carcinoma and ultraviolet light in skin cancer (Brash et al. 1991), or by exogenous mechanisms (Loeb 1989). To date, all the ras genes investigated have been activated by a single point mutation, altering the amino acid either at position 12 or around position 61 in the 189-amino acid ras protein product. In vitro mutagenesis has revealed that other codons in the human c-Ha-ras-1 gene where amino acid substitutions can also lead to a gene with transforming activity. Therefore, the alterations which "activated" the ras genes must therefore be critical determinants of the malignant phenotype in the tumor cells.

(c) Gene Amplification

The abnormalities result in the amplification of cellular genes: a single-copy proto-oncogene in a normal cell may be amplified up to 100 times in a tumor cell. The increased expression of the oncogene, resulting from its amplification, is believed to contribute to the malignancy. Examples now exist are multiple copies of Ki-ras in primary lung carcinomas and the amplification of the myc family in leukemic cells and tumors of neural origin (Varmus, 1984). Interestingly,the degree of amplification correlates with the stage of progression of the tumor: greater amplification correlates with advanced stage and a poor clinical prognosis (Schwab et al. 1984, Slamon et al. 1987).

Oncogene and Gap Junction Mediated Intercellular Communication

One form of intercellular communication is mediated by the membrane channels known as the gap junctions. These channels link two adjacent cells to allow the exchange of ions, nutrients and regulatory molecules. Gap junctions are constructed from hexamers of transmembrane proteins that called connexins. Three major forms of connexins, connexin 43, connexin 32, and connexin 26, were well characterized at the biochemical and molecular level.

The junctional communication has been implicated in the regulation of tissue homeostasis, cell growth and differentiation, as well as synchronization of tissue function and regeneration. Several modulators provided substantial evidence for an important role gap junctions might play in carcinogenesis. Many tumor cells showed absence of gap junctional coupling that the ability to communicate with normal cells via gap junctions.

The correlation between the neoplastic phenotype and the absence of gap junctional intercellular communication became more significant following the discovery that tumor promoters, e.g. phorbol esters, inhibit this important membrane mediated function (Yotti et al. 1979, Trosko and Chang, 1988). TPA (12-O-tetradecanoylphorbol-

13-acetate), a potent tumor promoter, is one of the major promoter shown to block intercellular communication. TPA is a structural analogue of diacylglycerol ,which is an endogenous activator of protein Kinase C generated by hydrolysis of phosphatidylinosotol 4,5-bisphosphate (PIP_2) by Phospholipase C. It was believed that the activated protein Kinase C can phosphorylate gap junction protein so that the communication is inhibited (Oh et al.1988). This evidence indicated that tumor promoters might act by blocking gap junctional coupling between normal cells and initiated cells thus facilitating clonal amplification of initiated cells.

Recently, inhibition of gap junctional communication has been also shown in oncogene transfected cells. Chang et al (1985) were the first to demonstrate that in NIH 3T3 cells transfected with v-src oncogene, GJIC was downregulated and this inhibition was correlated with increased protein Kinase C activation. Another oncogene product, p21 protein, generated by ras oncogene was also shown to abolish gap junctional communication in rat liver epithelial cells. Furthermore, it was found that cell-cell communication was blocked in v-raf/v-myc transfectant (Kalimi et al.1992).

Similar to protein kinase C, the raf protein is also a serine/threonine specific kinase. It was found that treatment cells with TPA, the activity of raf protein kinase increased as well as protein Kinase C(Morrison et al. 1992). Therefore, it was suspected that raf protein kinase might be able to downregulate GJIC in a manner similar to PKC. In the current investigations, v-raf transfectants were studied and the results will be discussed later.

Raf Oncogene

A.Viral raf oncogene

Cellular raf oncogene was first identified in its oncogenic form, v-raf, the oncogene of the acutely transforming virus 3611-murine sarcoma virus (3611-MSV) (Rapp et al. 1983).

The primary structure of v-raf was identified by Mark et al. in 1984. V-raf is expressed as a myristylated gag-raf fusion protein consisting of the amino-terminal 384 amino acids of Gag and the carboxyl-terminal 323 residues of mouse c-raf. (Rapp, 1983; Schultz, et al. 1985). Transfection of NIH3T3 cells with cloned 3611-MSV proviral DNA lead to highly efficient transformation and the recovered virus elicits tumors in mice typical of the 3611-MSV virus. It was also demonstrated that 3611-MSV was capable of transforming the rat liver epithelial cells (Garfield et al. 1988). However, the other experiment showed that cells infected with v-raf alone were only weakly tumorigenic and were morphologically more similar to control cells than those transformed with a combination of v-raf and v-myc, which were aggressively tumorigenic and showed increased refractivity and spindlings as well as decreased attachment (Hampton et al. 1990).

B.Localization and expression of raf gene family

The raf family of proto-oncogenes consists of three active members: A-raf-1, B-raf and C-raf-1. They are dispersed over different chromosomes and are expressed in different tissues. The mammalian C-and A-raf genes have 16 coding exons, which span 40 and 20kb, respectively. B-raf gene is larger and extends over 46kb (Bonner, T. et al. 1984). Raf-1 has been mapped to chromosome 3p25 in humans, a region frequently altered in small cell lung carcinoma, familial renal cell carcinoma, and ovarian caner(Kozak et al. 1984). In addition, c-raf-1 gene is ubiquitously expressed. Among the transcripts that have been detected in all cell lines and all normal and tumor tissues examined, the highest levels of expression observed was in striated muscle, cerebellum, and fetal brain (Storm, et al. 1990). A-raf was characterized by Huleihel in 1986 and localized to the x chromosome between p21 and q11 (Huebner et al. 1986). The A-raf gene is expressed at high levels in the urogenital system, including epididymis, ovary, kidney, and urinary bladder (Storm et al. 1990). The most recently identified member, B-raf, was discovered as a transforming gene in N1H3T3 cell transfection assays of human Ewing sarcoma DNA (Ikawa at al. 1988). B-raf expression is confined to fewer tissues than either c-raf-1 or A-

raf, with high message levels being observed in cerebrum and fetal brain. (Storm, 1990).

C. Raf protein

The raf oncogene family shows a high degree of evolutionary conservation. The c-raf-1 and A-raf genes encode cytoplasmic serine/threonine protein kinases of 68 and 74 kDa, respectively, which are 60% identical overall. The B-raf gene product has not yet been completely characterized. However, its predicted protein product is 76% identical to c-raf-1 and 74% identical to A-raf. The raf protein contains three structural domains (Heidecker et al. 1986), each of which is highly conserved in all raf genes.

The deduced amino acid homologies of these three regions are shown in Table 1. (Stephen et al. 1990). The first conserved region (CR1), located in the amino-terminal portion of the molecule, is a cysteine rich domain and contains the consensus sequences C-X2-C-X9-C-X2-C (Heidecker et al. 1989). This structure is similar to the ligand-binding domain of protein kinase C, CR1 comprises the presumed ligand binding site although a ligand has not yet been identified for Raf. A second highly conserved region, CR2, also located in the amino-terminal portion is rich in serine and threonine residues. The third conserved region, CR3, is located in the carboxyl-terminal portion of raf protein and is the serine/threonine kinase domain. The amino-terminal of the protein is thought to regulate the catalytic activity of the carboxy-terminal kinase domain.

D. Raf oncogene in Signal Transduction

There is considerable evidences that the raf family serine/threonine protein kinases have the hallmarks of signal transmitters involved in communicating between mitogen receptors at the cell surface and the nucleus. First, treatment of cells with several growth factors (eg. PDGF, EGF) increased both the level of phosphorylation and the intrinsic protein kinase activity of the raf protein (Morrison et al. 1989). Second, in PDGF-treated cells, Phospholipase C-γ associates with ligand-activated PDGF receptors in a complex that includes raf-1, PI-3 kinase, GTPase activating protein (GAP), and several src family

members (Kaplan, 1990). Third, treatment with phorbol esters to stimulate protein kinase C also leads to increased phosphorylation of raf protein and its kinase activity (Morrison, 1988). Furthermore, cells containing activated raf have an altered transcription pattern, and raf oncogenes behave as transcriptional activators for Ap-1/PEA 3-dependent promoters in transient transfection assays (Wasylyk et al. 1989). Finally, raf-transformed cells can proliferate in the absence of ras function (Smith et al). That is, the raf protein kinase can function in signal transduction independent of ras.

E. Activation of Raf protein kinase

There is ample evidence that raf protein plays an important role in transmitting signals from cell surface receptors to the nucleus, however, both the exact mechanisms by which raf is activated and downstream targets of the activated molecule remain to be elucidated. There are four possible models for the activation of raf serine/threonine kinase (proposed by Ping Li, et al, 1991). First, the kinase may be activated by a small second messenger molecule. The amino-terminal domain of Raf-1 is similar to the ligand-binding domain of PKC and thus may be a potential binding site for a lipid second messenger. In the second model, the raf kinase is activated by an upstream kinase, a so-called "Kinase kinase." The third model, the most direct route of activation, is via direct phosphorylation and consequent activation by a tyrosine kinase. Several experiments suggest this direct activation may occur. For example, tyrosine phosphorylation of Raf-1 is seen in vivo after PDGF treatment of fibroblasts, and a portion of the Raf-1 in the cell is found in a complexes with the activated receptor (Morrison et al. 1989). The forth model is that tyrosine phosphorylation brings about a transient change in Raf-1 conformation so that the raf kinase is activated.

Structural analysis of naturally occurring oncogenic raf (Parker et al.1986), and those that arise during the course of NIH3T3 cell DNA transfection assays (Stanton et al.1987: Ishikawa et al. 1987) indicate that the amino-terminal domain of Raf-1 is

important in the regulation of Raf-1 is important in the regulation of Raf-1 activity (Morrison,1990). Therefore, there are also some presumed mechanisms of Raf-1 activation by oncogenic and mitogenic events regarding regulatory domain (Morrison, 1990). Mutational analysis has revealed three oncogenic events that can generate a transforming Raf-1 protein: (1) removal of CR1 and CR2 by amino-terminal truncation of the protein; (2) disruption of CR2 by linker insertion; and (3) fusion of the intact protein to unrelated sequences. V-raf which expresses gag-raf fusion protein was thought to be activated by this mechanism. Mitogenic events can also activate Raf-1. As noted above, this occurs through phosphorylation of serine or tyrosine residues or through ligand binding to CR1.

F. Raf protein kinase and Protein kinase C

Protein kinase C was first identified in 1977 as a protein kinase (Inoue at al. 1977; Takai et al. 1977). It is also a serine/threonine kinase which plays roles in signal transduction pathways related to cell proliferation. The activation of protein kinase C is connected with phospholipase C. In other words, it was identified as an effector enzyme of the inositol phospholipid second messenger pathway and activated by diacylglycerol. The involvement of protein kinase C in tumor promotion was proved by the demonstration that protein kinase C is the major intracellular receptor for phorbol esters and other promoters. The serine/threonine kinase encoded by protein kinase C has a catalytic domain and a regulatory domain. The catalytic domain is localized to the carboxy-terminal half of the protein, which the regulatory domain comprises the amino-terminal half of the molecule, which includes the phorbol ester binding site. This structure is highly analogous to the raf kinases, which are also similar to protein kinase C in size. Therefore, there is a strong analogy between activation of protein kinase C catalytic activity by binding ligand to its amino-terminal regulatory domain and activation of raf transforming potential by deletion of its amino-terminal domain as discussed above.

Since there is structural similarity between raf protein kinase and protein kinase C and since both of them play important roles in signal transduction pathway, it is considered that these two kinases are functionally interrelated. One evidence is that treatment of cells with phorbol esters to stimulate protein kinase C also leads to increased phosphorylation of raf protein and its kinase activity. There are two hypotheses regarding the correlation between protein kinase C and raf protein kinase. First, the path of activation is parallel. They may be activated separately by kinase including recruitment of second messengers derived from tyrosine-stimulated lipid turnover and direct phosphorylation by tyrosine and serine/threonine-specific protein kinases. Second, raf protein kinases are downstream to protein kinase C. The PDGF receptor is associated with phospholipase C, which appears to be a substrate for phosphorylation by the receptor tyrosine kinase. The activity of PLC may be stimulated by phosphorylation, leading to increased hydrolysis of phosphatidylinositol 4, 5-bisphosphate (PIP_2) and production of the second messengers diacylglycerol (DAG) and inositol 1, 4, 5-triphosphate (IP_3). The combination of diacylglycerol and calcium will activate protein kinase C. The activated PKC then activate raf protein which transduce signal to the nucleus.

G. Raf oncogenes in carcinogenesis

The 3611-MSV retrovirus which transduce the raf gene has been studied in carcinogenesis. Newborn mice treated with the 3611-MSV virus developed severe erythroleukemia after 4-12 weeks and only occasionally do myelogenous and lymphoblastic neoplasms occur simultaneously with the erythroleukemias. (Klinken, 1991). The other experiments have been done in human tumors. A NIH 3T3 transfection assay using DNA from a cell line established from a radioresistant human larynx carcinoma led to isolation of a 5'-truncated human Raf-1 gene. (Kasid, et al. 1987).

Since Raf-1 has been mapped to chromosome 3P25 it has been of particular interest to study the role it plays in tumors that show consistent or high frequency loss of 3P, such as

small cell lung carcinoma (SCLC), renal cell carcinoma, ovarian carcinoma, and mixed parotid gland tumors. Comparing 11 samples of small cell lung cancer to the corresponding normal tissue, a consistent loss of chromosome 3P alleles was found.(Rapp, et al. 1988). In addition, among 73 human lung cancer cell lines were examined, a highly significant loss of one Raf-1 allele was detected in all 42 SCLC cell lines. These results demonstrated that one allele of Raf-1 is consistently lost in small cell lung carcinoma. (Rapp, et al. 1988).

MATERIALS AND METHODS

The viral raf plasmid cDNA (Figure 1) used in this research was kindly provided by Dr. Rapp who is from Laboratory of Viral Carcinogenesis, National Cancer Institute, Frederick, Maryland. The cellular and viral plasmid DNAs were then transfected into E. coli strain, DH5α . The following step is to isolate large amount of those plasmid DNAs from bacterial using alkaline lysis method. The DNAs were used to be transfected into WB-F344, rat liver epithelial cells, using lipofectin. Because the constructs of raf oncogenes contained neomycin resistant gene, neomycin was used to select those cells which contain the integrated raf genes. The DNAs, RNAs and proteins were isolated from the transfectants to study the level and the expression of raf gene and raf protein using northern blot analysis and western blot analysis. Scrape Loading/Dye Transfer Technique was applied to study the effect of viral oncogene in intercellular communication. The method of anchorage independent growth(AIG) was used to assess the tumorigenicity of v-raf transfectants. These methods will be described in greater detail later.

Preparation of competent E. coli

A colony of E. coli strain DH5α was picked up by a sterile wire from LB plate (1% NaCl, 1% tryptophan, 0.5% yeast extract, 1.5% Agar) and grew in a 5 ml of LB medium in 15 ml polystyrene tubes (Falcon 2099). After incubating the culture overnight at 37°C, 1 ml of the culture was transferred to a 25 ml LB medium in a sterile, disposable 50 ml polypropylene tubes (Falcon 2098) and incubated at 37°C. The OD_{550} was checked every 20-30 minutes until the value of OD_{550} was equal to 3.0. The culture was cooled on ice for 10 minutes and then centrifuged at 3000 rpm for 10 minutes at 4°C. the cells were resuspended in 25 ml of ice-cold 100 mM $CaCl_2$ and centrifuged again. The cells were resuspended in 2.5 ml of ice-cold 100 mM $CaCl_2$. These cells were then used for

transformation and stored at -70°C containing 15% glycerol.

Transformation of competent cells with raf plasmid cDNA

One hundred microliter of competent cells from above were transferred into a 17x100mm polypropylene tube. Twenty nanograms of raf plasmid DNA isolated as described below was added to the tube. The tube was then placed on ice for 30 minutes with occasional mixing. After heat shock at 42°C for 2 minutes, the cells were kept at room temperature for 10 minutes. The transformed cells were then transferred to 1 ml of LB medium and incubated at 37°C for 1 hour. One hundred microliter of the culture was streaked on a LB plate containing 50 ug/ml of Ampicillin and incubated at 37°C overnight.

Small-scale preparations of plasmid DNAs by alkali lysis method

A single bacterial colony from above step was transferred into 2 ml of LB medium containing 50 ug/ml of Ampicillin in a 15 ml tube and incubated overnight at 37°C with vigorous shaking. A 1.5 ml of the culture was poured into a 1.5ml eppendorf tube and centrifuged at 12,000 rpm for 30 seconds at 4°C. The medium was removed by aspiration, leaving the bacterial pellet as dry as possible. The bacterial pellet was resuspended in 100 ul of ice-cold solution I (50 mM glucose, 25 mM Tris.Cl (pH8.0), 10 mM EDTA (pH8.0)) by vigorous votexing. Then 200 ul of freshly prepared solution II (0.2N NaOH, 1% SDS) was added followed by 150 ul of solution III (3M NaOAC, pH5.2) and stored on ice for 30 minutes. The tube was centrifuged at 12,000 rpm for 10 minutes at 4°C and the supernatant was transfered to a fresh tube. Two volumes of ice-cold ethanol was added to precipitate the double-stranded DNA. After 15 minutes at -70°C, the tubes centrifuged at 12,000 rpm for 10 minutes at 4°C. The supernatant was removed by gentle aspiration and the pellet was washed by 70% ethanol. The air dried nucleic acids were redissolved in 20 ul of TE (10 mM Tris, 1mM EDTA pH8.0).

Large-scale preparations of plasmid DNA by alkali lysis method

500 ml of LB containing 50 ug/ml ampicillin in a 2-liter flask with 1 ml of the late-log-phase culture was incubated at 37°C overnight (12-16 hours) with vigorous shaking (250 cycles/minute on a rotary shaker). The bacterial cells from the 500 ml culture were harvested by centrifugation at 10,000 rpm for 10 minutes at 4°C. The bacterial pellet was resuspended in 10 ml of solution I followed by adding 20 ml of freshly prepared solution II described above. Fifteen milliliters of ice-cold solution III were then added and mixed by inverting several times. After keeping on ice for 30 minutes, the bacterial lysate was centrifuged at 10,000 rpm for 10 minutes in a Sorvall RC2B-high speed centrifuge using a SS-34 rotor. Two volumes of ethanol were added into the supernatant and stored in a freezer(-20°C) for 1 hour. The nucleic acids were recovered by centrifugation at 10,000 rpm for 10 minutes at 4°C. The pellet was rinsed by 70% ethanol and dissolved in 1.5 ml of TE(pH8.0). [The solution I, II, III are the same as the small-scale preparation].

Purification of plasmid DNA by precipitation with polyethylene glycol(PEG)

One volume of 5 M LiCl was added to the DNA isolated above and stored on ice for 20 minutes. After centrifugation for 15 minutes at room temperature, 1 volume of isopropanol or 2 volumes of ethanol was added to the supernatant and nucleic acids were repelleted by centrifugation at 10,000 rpm. The pellet was rinsed with 75% ethanol and resuspended in TE (pH 8.0). These DNAs were digested with 20 ug/ml of Rnase for 1 hour. One volume of 13% PEG 8,000 and 1.6M NaCl were added to remove protein and RNA. The pellet was resuspended in 200 ul TE(pH8.0) after kept on ice and span 15 minutes at room temperature. The DNAs were extracted once with 1 volume of phenol, once with one volume of phenol/chloroform and once with one volume of chloroform to remove the RNAs and other proteins. The supernatant was precipitated by adding 1 vol.

of isopropanol and 0.1 volume of 3M sodium acetate (pH5.2). The pellet obtained by centrifugation was rinsed with 75% ethanol and air dried . The nucleic acids were dissolved in 500 ul of TE (pH8.0). An aliquot of this solution was diluted 10-fold with distilled water and the absorbance was recorded using a Gilson UV-Spectrophotometer. The ratio of OD_{260} : OD_{280} usually ranged from 1.8-2.0 indicating DNA free of protein contamination.

Cell culture

The WB-F344, rat liver epithelial cells and other raf-transfected cells were routinely grown in a modified Eagles' MEM, which contains Earle's balanced salt solution with a 50% increase of vitamins and essential amino acids, a 100% increase of non-essential amino acids and 1 Mm sodium pyruvate, 5.5 Mm glucose, 14.3 Mm NaCl, 11.9mM $NaHCO_3$, pH7.3] This medium was supplemented with 10% fetal bovine serum (GIBCO, BRL) and 50 ug/ml gentamicin (Quality Biological Inc. MD). For transfected cell line, 400 ug/ml of neomycin was added as selective agent. The cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air and subculture whenever they were confluent.

Transfection of WB cells with viral raf oncogene

DNA transfection is commonly used to introduce DNA into eukaryotic cells for the study of gene expression. A variety of DNA transfection procedures have been developed, while in this research, the cationic liposome-mediated transfection method was used. Lipofectin reagent (BRL, cat. No. 8292SA) is a 1:1 (w/w) liposome formulation of the cationic lipid N-(1-(2,3-dioleyloxy) propyl)-N,N,N-trimethylammonium chloride (DOTMA) and dioleyl phosphatidyl-ethanolamine (DOPE) in membrane-filtered water.

This reagent is taken up phagocytically by cells and interacts spontaneously with DNA to form a lipid-DNA complex. The fusion of the complex with tissue culture cells results in expression of the DNA.

In this method, WB cells were seeded in three 60-mm tissue culture dishes in 5 ml of D medium (described above) supplemented with 10% fetal bovine serum. These cells were incubated at 37°C in 5%-CO_2 environment until they are 40-50% confluent. For each 60-mm dish, 10ug of raf plasmid DNA and 50ug of lipofectin reagent was diluted to 1.5 ml of serum free medium separately. 1.5 ml each of the DNA and the lipofectin reagent dilutions were combined in a polystyrene tube and mixed gently . Then this complex was added to the dishes after washing the cells twice with 3 ml serum-free medium. The cells were incubated for 16 hrs at 37°C in CO_2 environment. 3 ml of growth medium supplemented with 10% fetal bovine serum were added to the cells. After 12 hrs growth, those cells were split into three of 100 mm cell culture dishes and started selection the following day.

Since the viral raf plasmid DNA contain neomycin resistant gene, G418 (GIBCO , NY) was used to select the clone which contain viral raf oncogene, 300 ug/ml of G418 was used for selection.

Scrape-Loading/Dye Transfer Technique to study GJIC

Cells were grown to confluence in 35 mm plates. The plates were washed with PBS several times. 2 ml of Luciffer yellow solution (0.5 mg/ml in PBS; Sigma, cat. No. L0259) was added to the plates and three scrape lines were made on the cell monolayer with a surgical blade. After 3 minutes to allow dye uptake and transfer at room temperature, the cells were washed several times with PBS and fixed in 10% phosphate-buffered formalin. Photomicrographs of the fluorescence images of the dye loaded cells were taken to determined the extent of dye transfer of the clones.

Isolation of total RNA from cells

Total RNA was isolated by the single-step method using Acid Guanidinium Thiocyanate-Phenol-Chloroform Extraction (Chomczynski, et al. 1987), as described below:

Confluent cultures of cells in 25 cm^2 flasks were rinsed with PBS and lysed in 0.75 ml of solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH7.0; o.5% sarcosyl, 0.1 M 2-mercaptoethanol) after removing the PBS. The lysate was subsequently transferred to a 2.2-ml eppendorf tube. Sequentially, 75 ul of 2 M sodium acetate, pH4.0, 0.75 ml of phenol, and 200 ul of chloroform-isoamyl alcohol mixture(49:1) were added, with thorough mixing by votex after the addition of each reagent. Samples were centrifuged at 14,000 rpm for 15 minutes at room temperature. The aqueous phase was transferred to a fresh tube, mixed with 1 ml of isopropanol, and placed at -20^oC for 1 hour to precipitate RNA. The tubes were centrifuged at 10,000 rpm for 30 minutes at room temperature, 200 ul of 4 M LiCl was added to the pellet with slowly shaking over a 20-minute period. Sedimentation at 14,000 rpm for 10 minutes was again performed and the resulting RNA pellet was dissolved in 200 ul of 0.5% SDS. The RNAs were extracted once with 200 ul of chloroform. The aquous phase was precipitated using 20 ul of 3M sodium acetate, pH5.2 and 400 ul of ethanol. After centrifugation, the resulting RNA was washed with 75% ethanol and subsequently dissolved in 20-50ul of 0.5% SDS. The concentration of the RNAs were measured by reading OD_{260}.

Northern Blot Analysis

I. Electrophoresis of RNA through gels containing formaldehyde

One gram of agarose was added to 100 ml of DEPC-treated ddH_2O and boiled in microwave until the volume was reduced to about 70 ml. Ten milliliters of 10X MOPS (3-[N-Morpholino] propane-sulfonic acid) and 18 ml of formaldehyde were then added to

the flask to prepare a 1X MOPS, 3%-formaldehyde gel. The gel solution was poured into a gel tray to make about 5 mm-thick gel. After polymerization in about 1 hour, the running buffer (1X MOPS, 3% formaldehyde in DEPCddH_2O) was poured into an electrophoresis box to cover the gel with the buffer. Twenty micrograms of each sample RNA in 20 ul of sample buffer (50% formamide, 1X MOPS, 6% formaldehyde, 1 ul of tracking dye, 1 ug/ml of Ethidium Bromide) was heated at 68°C for 10 min. and then chilled on ice . The samples were loaded into wells and electrophoresed at 80 volts for 4 hours, or 10 volts overnight with buffer recirculation

II. Northern Blot

After electrophoresis, the gel was soaked in DEPC-treated ddH_2O for 45 minutes twice. A picture of the gel was photographed by using ultraviolet illumination. The gel was soaked in 20X SSC for 45 minutes with agitation and set up for capillary transfer of RNA to nylon membrane. The RNAs were transfered from the gel to a nylon membrane (Hybond^{TM}-N, 0.45 micron; Amersham corporation) which was pre-wet in ddH_2O and soaked in 20X SSC. After transfer overnight, the membrane was washed in 5X SSC briefly and dried in the air for 10 minutes. The membrane then was baked at 80°C in a vacuum owen(Fisher Scientific) for two hours and exposed to UV light(254 nm) to cross link the RNA to membrane and prepared for hybridization with ^{32}P-labelled c-DNA probes.

III. Hybridization and Autoradiography

(a) Preparation of α-^{32}P labelled c-DNA probes.

The raf-1 gene is 290 bp fragment (Oncor, cat. P2115) and the connexin 43 cDNA was isolated from Blue-G2A plasmid. Probes were made using a random primed DNA Labelling kit (Molecular Biology, Bochringer mammheim).

Fifty nanograms (18ul) of the DNA was denatured by heating for 10 minutes at 95°C and subsequently chilled on ice. The following compoents were added to the tube to set up

the reaction: 6 ul of dATP,dGTP,dTTP mixture (1:1:1 mixture of each dNTP), 4 ul of reaction mixture (random hexanucleotides in 10X reaction buffer), 10 ul of [α-^{32}P]dCTP, 50uCi, 3000 Ci/mmde and 2 ul of klenow enzyme. The reaction mixture was incubated for 1 hour at 37^{o}C and the reaction was terminated by adding 10 ul of 0.2 M EDTA,pH8.0. The product of this reaction was purified by Bio-spin columns (Bio-Rad ,Cat. No. 732-6006).Before adding the mixture to the column, the column was warmed to room temperature and was inverted several times to resuspend the settled gel. The top cap of the column as well as the bottom stopper to allow the excess buffer to drain by gravity. The column was then placed in a collection tube and centrifuged for 2 minutes in a horizontal rotor at 1,100Xg. The sample was applied very carefully to the center of the column, allowing the liquid to drain into the gel bed between successive drops of the sample. The column containing the applied sample was placed in a collection tube and centrifuged for 4 minutes at 1,100Xg. The purified labelled-probe in the collection tube was denatured by heating for 10 minutes at 95^{o}C and chilled on ice. This probe was then used for hybridization.

(b) Hybridization with labelled c-DNA and autoradiography

The membrane containing RNA(Northern blot) was pre-hybridized in a sealing bag containing 10 ml of prehybridization buffer (5X SSC, 5X Denhardt's, 0.5% SDS, 250 ug/ml Herring sperm DNA, 5% Dextran sulfate) at 65^{o}C for 1 hour. For hybridization, the ^{32}P-labeled c-DNA probe was added to the prehybridization buffer and incubation was continued overnight. The membrane was then washed twice with 2X SSC, 0.1% SDS for 10 minutes at room temperature and twice with 0.5X SSC, 0.1% SDS for 30 minutes at 65^{o}C. Autoradiographies were done at -70^{o}C using Kodak X-OMAT and Kodak X-OMAT regular screen.

After exposure, the film was processed in an automatic X-ray film processor (Department of Radiology, Michigan State University).

Western Blot Analysis

I. Isolation Protein from cells

Confluent cultures in 25 cm^2 flask washed with PBS several times and 0.5 ml of lysis buffer (20% SDS, 0.1 M Tris, pH7.5, 20 mM EDTA, 5% 2-mercaptoethanol and 1 mM PMSF) was added to lyse the cells. The cell lysate was transferred into 2.2-ml ependrof tube and sonicated for 30 seconds. Then the protein samples were stored at -20°C.

II. SDS-Polyacrylamide Gel Electrophoresis of Proteins

SDS-PAGE was done according to Laemmli using a discontinuous buffer system (1970). The apparatus was bought from Bio-Rad Inc. The glass plates were assembled according to the manufacturer's instructions. 10 ml (for two gels) of 10% separating gel (4 ml of ddH_2O; 2.5 ml of 1.5 M Tris, pH8.8; 100 ul of 10% SDS; 3.33 ml of Bis:Acrylamide (0.8:30); 50 ul of 10% Ammonium Persulfate; 2.5 ul of N,N,N,N-Tetramethylethylenediamine (TEMED) was poured into the gap between the glass plates and overlay the acrylamide solution with isopropanol to prevent oxygen from diffusing into the gel and inhibiting polymerization.

After polymerization was completed (about 30-45 minutes), the isopropanol was poured off and a clean comb was inserted into the space between two glass plates. 5 ml (for two gels) of 4% stacking gel (3 ml of ddH_2O, 1.3 ml of 0.5 M Tris, pH 6.8, 50 ul of 10% SDS, 650 ul of Bis-Acrylamide (0.8:30), 25 ul of 10% Ammonium Persulfate, 4 ul of TEMED) was filled into the spaces of the comb completely.

While the stacking gel was polymerizing, 20 ul of samples were prepared in 1X SDS gel-loading buffer. After polymerization of the stacking gel, the comb was removed carefully and the gel was mounted in the electrophoresis apparatus. Tris-glycine electrophoresis buffer (25 mM Tris, 250 mM glycine, 0.1% SDS) was added to the top and bottom reservoirs and the samples were loaded into the wells using microliter-pipet tips. Electrophoresis was done at a constant voltage of 100/cm. The gel was run until the

bromophenol blue reached the bottom of the resolving gel (about 2 hours). The gels werecarefully removed from the glass plate and used for western blotting.

III. Transfer of Proteins from SDS-Polyacrylamide Gels to Polyvinylidene Difluoride (PVDF) Membranes.

When the SDS-polyacrylamide gel was approaching the end of its run, one piece of Immobilon PVDF membrane and four pieces of whatman 3MM paper were cut to the exact size of the SDS-polyacrylamide gel.

The following precautions were taken to ensure the transfer of proteins from the gels to the PVDF membrane. First, the 3MM paper was put in transfer buffer to allow it to wet and the PVDF membranes need to be put in methanol first followed by transfer buffer. Second, a glass pipet was used as a roller to squeeze out any air bubbles between gel and membrane. Third, the 3MM papers and membrane were the same size as gel, otherwise, there was a good chance that the overhanging deges of the paper and the filter will touch, causing a short circuit that will prevent the transfer of protein from the gel.

A voltage of 20 Volt/cm was applied to the gel to transfer overnight. The gel was stained with 0.1% Coomassie Brilliant blue in methanol:H_2O/Aceteic Acid (1:1 V/V) while the membrane was stained with Ponceau S.

IV. Immunoblotting

The non-specific binding sites were blocked by soaking PVDF membranes for 2 hours at room temperature in phosphate buffered saline (PBS)/3% nonfat dry milk. The membranes were incubated for overnight at 4^{o}C with anti-mouse raf-1 kinase which was diluted to 3 ug/ml in 5 ml of buffer containing 4% nonfat dry milk, 40 nm Tris and 0.1% Tween 20. The membranes were then washed four times with the same buffer. The membrane was incubated with 1/500 dilution of biotinlayted anti-mouse Ig (Species-specific F(ab') fragment) for 1 hour and followed by washing three times with the same buffer. The following step was to incubate the membrane with 1:2500 dilution (in 40 mM tris, 0.1% tween 20, 4% milk and 0.5 M NaCl) of alkalinephosphatase-conjugated

streptavidin for one hour. Sequentially, the membranes were washed four times (each 5 minutes) with 20 mM tris, 0.1% tween 20 and 0.5 M NaCl. The last step was to develop the color reaction at room temperature with 5-bromo-4-Chloro-3-inclolyl phosphate (BCIP)/nitro blue tetrazolium (NBT) color development solution: One tablet of NBT was dissolved in 30ml of alkaline phosphatase buffer (0.1 M tris, 0.1 M NaCl and 5 mM MgCl2 , pH9.5) and 100ul of BCIP was added to the buffer just before color development.

Procedure for Anchorage Independent Growth (AIG)

3.3% of Agarose was prepared in PBS and boiled using microwave oven, then was kept in 45^{o}C waterbath. 2.55 ml of medium (D medium supplemented with 10 % fetal bovine serum) was warmed up at 39^{o}C and mixed with 0.45 ml of 3.3% agarose solution with a warm pipet to make 0.5% hard agar layer (0.5%) in 60 mm plate. This plate was put in incubator to let it solidify about one hour. Then, the soft agar layer (0.33%) with cells was prepared as follows: 2.2 ml of medium 0.3 ml of 3.3% agarose and 0.5 ml of cells in medium ($1X10^4$ cells/ml, Table 2) were mixed well and were inoculated on top of hard agar layer (the medium was warmed up in 39^{o}C water bath). After these plates were incubated at 37^{o}C for three days, 3 ml of liquid medium was added and renewed every three days. The cells were grown for four weeks and stained with 1 mg/ml of the tetrazolium salt, 2-(P-iodophenyl)-3-(nitrophenyl)-5-phenyltetrazolium chloride (Sigma I-8377) in 0.9% sodium chloride for 24 hrs at 37^{o}C.

RESULTS

A. Selection of v-raf transfectant

The construction of v-raf plasmid DNA contains neomycin resistant gene. Neomycin is a bacterial antibiotic which interfere with prokaryotic ribosomes, while mammalian cells are not affected by neomycin. The analogue, G418, now available through Gibco as Geneticin will affect eukaryotic ribosomes. The neo^R gene code for a phosphotransferase which inactivates the G418. Therefore cells which carry the plasmid and express this resistance marker (v-raf integrated) can survive in selective media. 300 ug/ml of Geneticin (Gibco) was used to select v-raf transfectant. The total number of clones resulted from the selection was about 20/60mm plate .

B. In vitro Morphology

Among the clones obtained from selection, only one clone was found to be morphologically transformed (WB-v-raf I). The morphplogy of the other clones were similar to the untransfected parental rat liver epithelial cells (Figure 2). The transformed cells showed spindle-shaped morphology and loss of contact inhibition of growth (Figure 3 and Figure 4) . Interestingly, the transformed cells reverted to normal morphology similar to parental cells after a few passages.

C. Expression of v-raf mRNA

Northern blot analysis of raf transcripts indicated the presence of a 3.3-kilobase mRNA for endogenous c-raf in each cell line. Whereas only two out of six v-raf-transfected clones (WB-v-raf A2 and WB-v-raf I) were found to have an additional 8.1-kilobase band which is v-raf mRNA (Figure 5). It was appeared that the clones which had v-raf-expression, also had increased level of endogenous c-raf-expression. The WB-v-raf I cells which lost transformed morphology at latter passage were found to have lose v-raf

mRNA (Figure 6).

D. Intercellular Communication

(a) Scrape Loading/Dye Transfer

Using the technique of scrape loading/dye transfer, the relative levels of gap junctional intercellular communication of all v-raf transfectant clones were determined. It showed that WB-v-raf A2 clone had the same level of communication as parental WB cells and the plasmid control cells ($G418^r$) (Figure 7). While the WB-v-raf I showed reduced communication (Figure 7). WB-v-raf I treated with TPA (100 ng/ml) overnight showed reduced GJIC compared to the same cells without TPA treatment. In the same experiment, WB-v-raf A2, the parental WB cells and the $G418^r$ plasmid control showed extensive GJIC (Figure 8).

(b) Expression of connexin 43 mRNA

Northern blot analysis of connexin 43 transcripts indicated the presence of a 3.1 kilobase mRNA in each cell line. The v-raf transfected G418-resistant clone with transformed morphology (WB-v-raf I) showed the same level of connexin 43 mRNA as did the wild type WB cells (Figure 9). It suggested that the reduction of intercellular communication might be due to posttranslational level.

E. Expression of raf p74 protein kinase in raf transfectant

Western blot analysis of v-raf transfectants using monoclonal anti-c-raf antibody showed that only the protein isolated from early passage of the morphologically transformed clone (WB-v-raf I) reacted with the antibody and showed raf p74 protein specific band (Figure 10).

F. Anchorage Independent Growth (AIG)

After the cells plated in soft agar for three weeks, the WB-v-raf I (with transformed morphology and expression of v-raf mRNA and protein) and WB-v-raf A2 (with normal morphology and expression of v-raf mRNA) were found to be able to grow

in soft agar, while the wild type WB, WB-neo^r cells, WB-v-raf A1 (a G418$\underline{r}$ clone without the expression of v-raf mRNA) cells can not grow (Figure 11). It is noticeable that WB-v-raf I clone has higher frequencies of AIG$^+$ colonies than WB-v-raf A2. The latter, however, was found to form larger colonies (Figure 12).

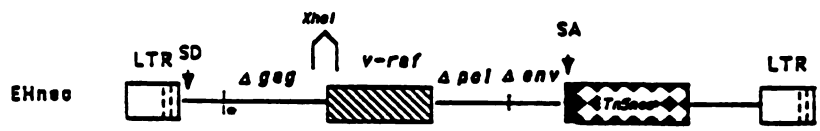

Figure 1. Construction of v-raf in EHneo plasmid DNA. Neo-vectors contain the Tn5 neomycin-resistance gene, which is expressed as a subgenomic RNA after splicing at an RSV promoter inserted in front of it. Special features of the constructs are as follows: SD and SA are splice donor and acceptor sites,respectively;the asterisk indicates the presence of the myristilation site. (From U.R.Rapp et al. Cold Spring Harbor Symposia on Quantitative Biology LIII: 173- 183,1988).

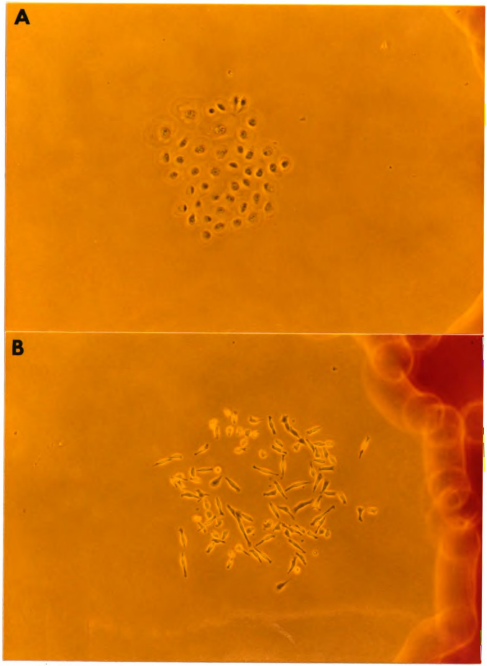

Figure 2. Single clone resulted from selection
A. This clone showed same morphology as wild type(100X)
B. This clone showed morphological transformation(100X)

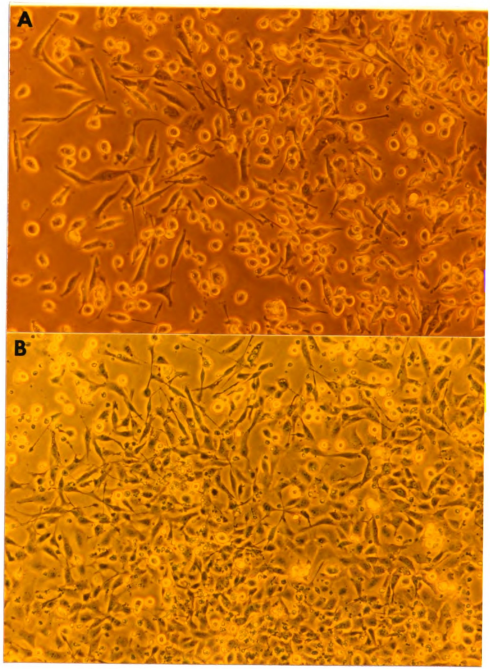

Figure 3. Morphological features of WB-v-raf I clone.

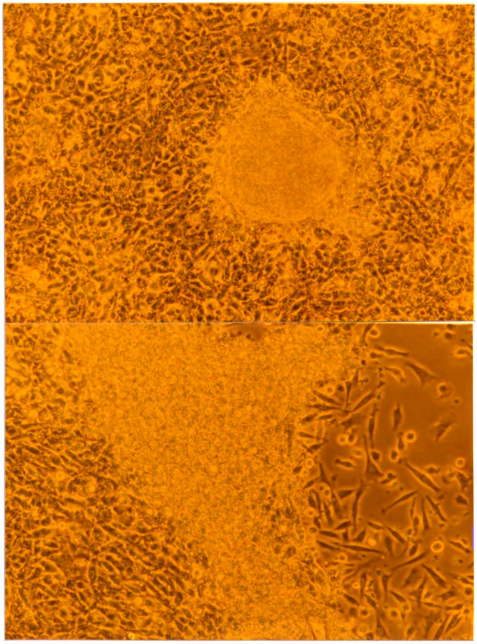

Figure 4. Foci formed by WB-v-raf clone which showed loss of contact inhibition.

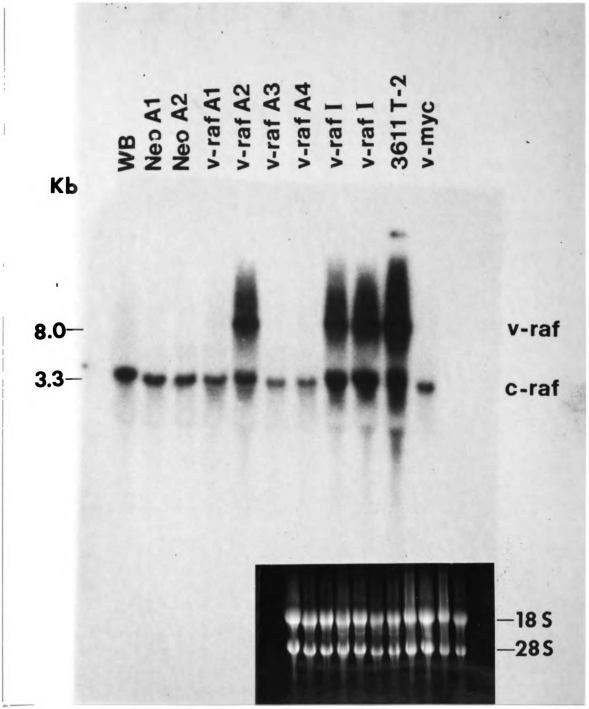

Figure 5. Northern blot analysis of v-raf transcripts in the various cell lines. Twenty micrograms of total RNA were separated on a 1% agarose/2.2 M formaldehyde gel, blotted onto nylon, and hybridize with ^{32}P-labeled v-raf probes.

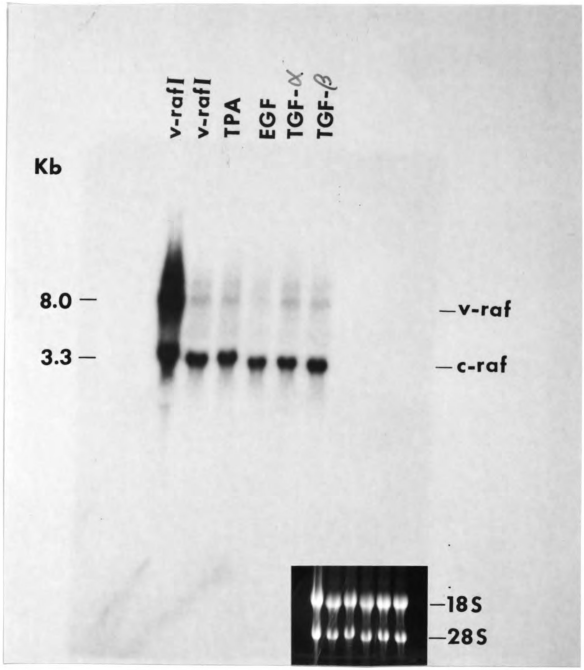

Figure 6. Northern blot analysis of v-raf transcripts in WB-v-raf I cells. Lane 1. WB-v-rafI transformed cells. Lane 2. WB-v-raf I revertant cells. Lane 3. WB-v-raf I cells treated with 100ng/ml of TPA for 48 hrs. Lane 4. WB-v-rafI cells treated with 100ng/ml of EGF for 48 hrs. Lane 5. WB-v- rafI cells treated with 100ng/ml of TGF-α for 48 hrs. Lane 6. WB-v-rafI cells treated with 1 ng/ml of TGF-ß for 48 hrs.

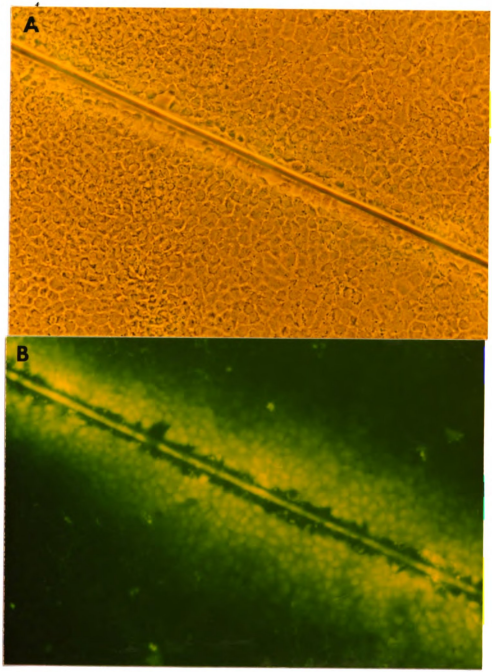

Figure 7(a). Gap junctional mediated dye transfer detected by Scrape-Loading/Dye ransfer.

A. Phase-contrast micrograph of WB-F344 cells.

B. Fluorescent micrograph showed Lucifer Yellow transfer to contiguous cells.

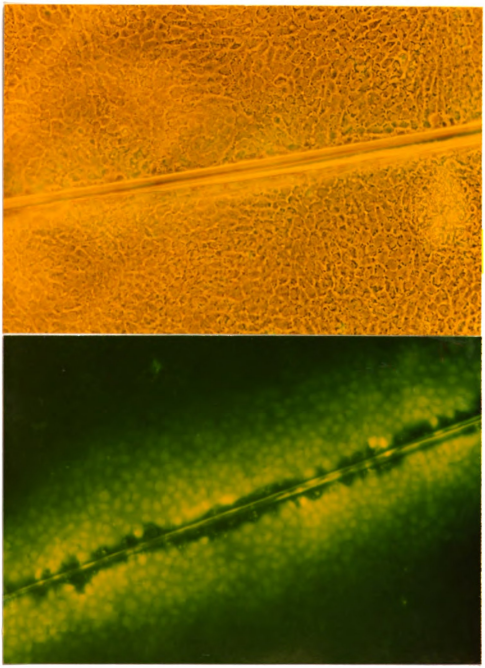

Figure 7(b). Gap junctional mediated dye transfer in pSVneo transfected WB cells.

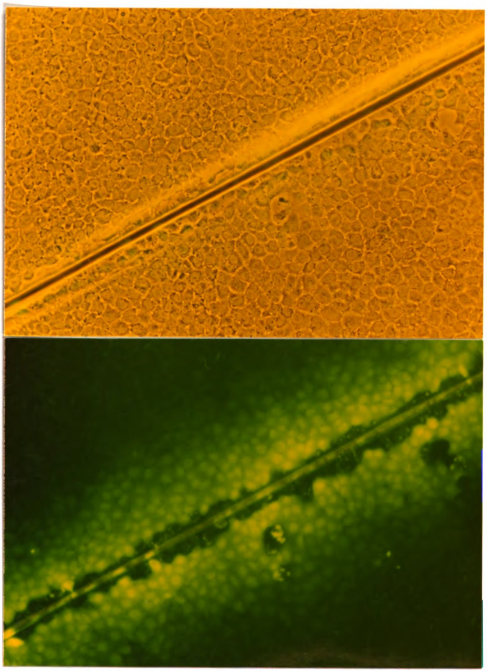

Figure 7(c). Gap junctional mediated dye transfer in WB-v-raf A1 cells.

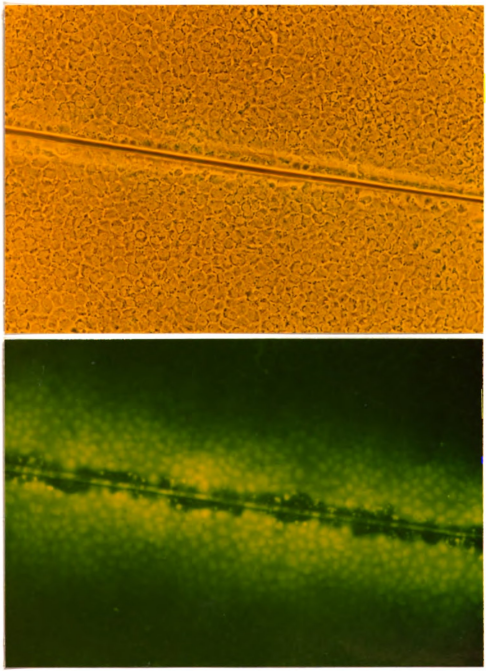

Figure 7(d). Gap junctional mediated dye transfer in WB-v-raf A2 cells.

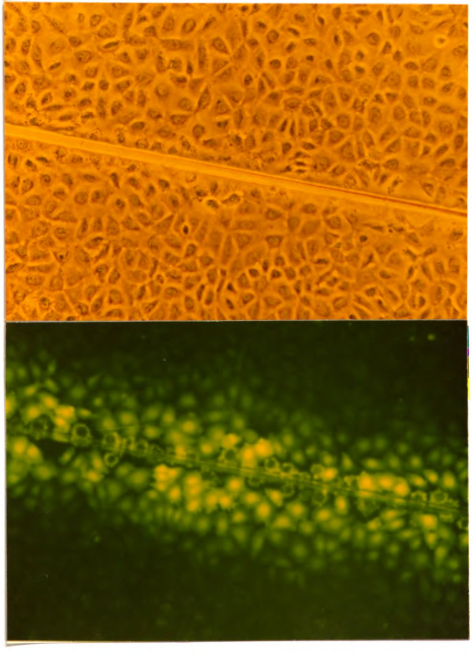

Figure 7(e). Gap junctional mediated dye transfer in WB-v-raf A3 cells.

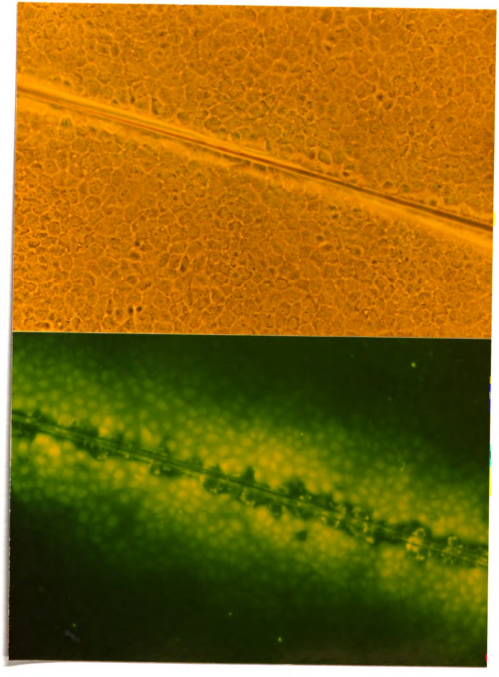

Figure 7(f). Gap junctional mediated dye transfer in WB-v-raf A4 cells.

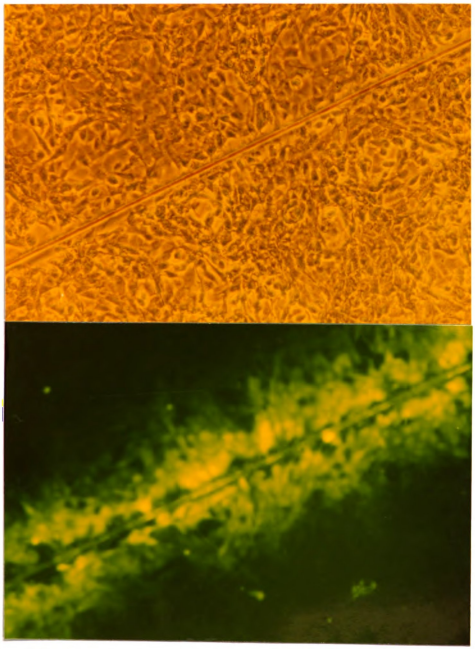

Figure 7(g). Gap junctional mediated dye transfer in WB-v-raf I cells.

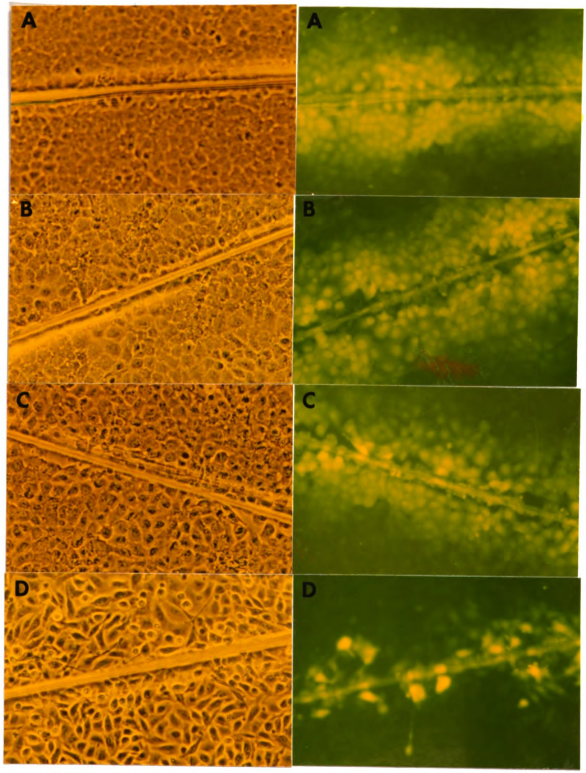

Figure 8. Gap junctional mediated dye transfer. Cells were treated with 100 ng/ml of TPA for 24 hrs. A. WB parental cells B. pSVneo plasmid control cells C. WB-v-raf A2 cells D. WB-v-raf I cells.

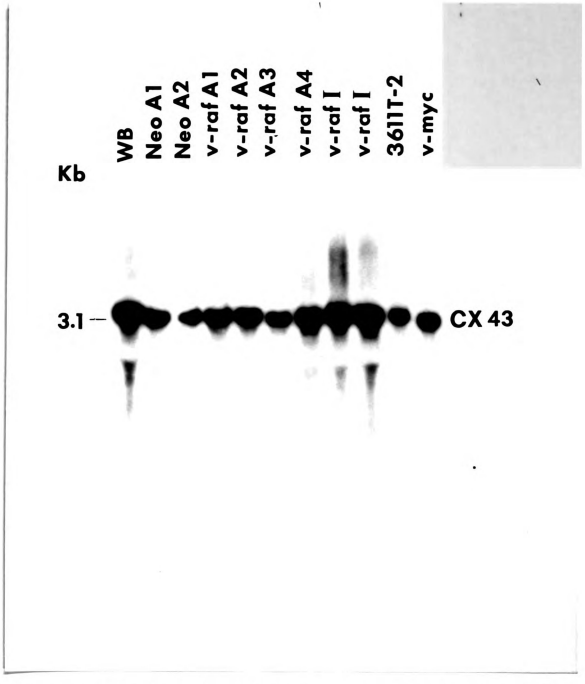

Figure 9. Northern blot analysis of connexin 43 transcript in the various cell lines. Twenty micrograms of total RNA were separated on a 1% agarose/2.2 M formaldehyde gel, blotted onto nylon, and hybridize with ^{32}P-labeled cx43 cDNA probes.

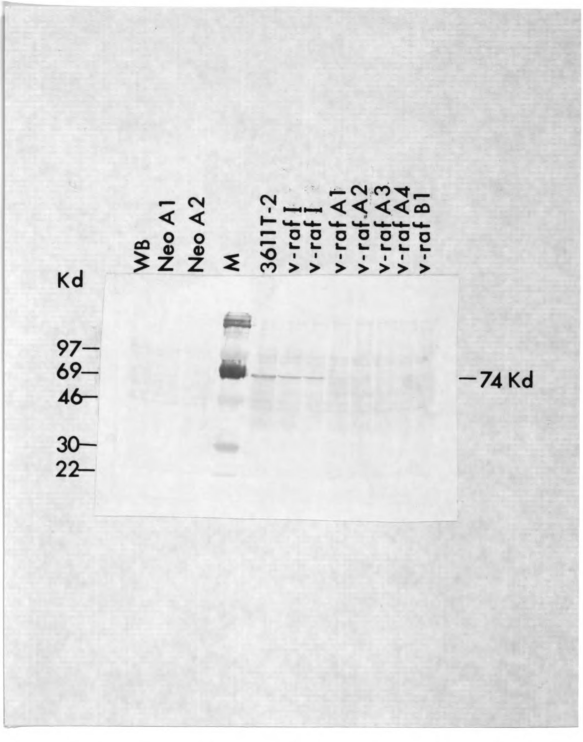

Figure 10. Western blot analysis of proteins in cells transfected with v-raf and pSV2neo. Total lysates of cells were separated by SDS-PAGE, transferred to PVDF membrane, and probed with anti-raf kinase. Bands were visualized with alkaline phosphatase.

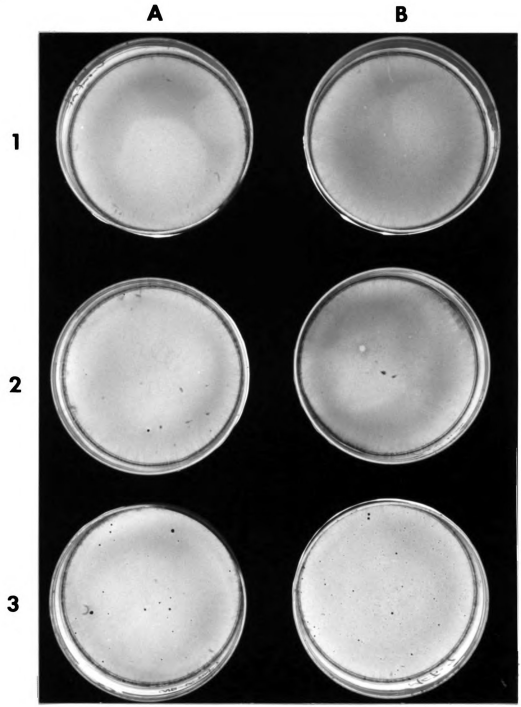

Figure 11. Anchorage independent growth (AIG) of the various cell lines. A-1: WB-v-raf A1 clone A-2: WB-v-raf A2 clone A-3: WB-v-rafI clone B-1: WB parental cells B-2: pSVneo plasmid control B-3: MCF7 positive control cells.

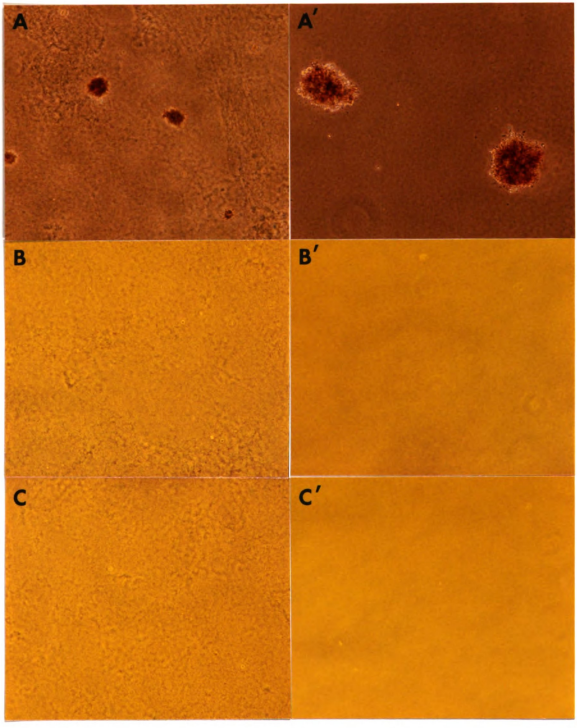

Figure 12(a). Anchorage-independent growth (AIG) of the various cell lines. Five thousand of cells were grown in 0.33% soft agar layer. Colonies were stained with 1mg/ml of 2-(p- iodophenyl)-3-(nitrophenyl)-5-phenyltetrazolium chloride in 0.9% NaCl for 24 hours at 37°C. A.MCF-7 (positive control,40X) A'.MCF-7(positive control,100X) B.WB parental cells(40X) B'.WB(100X) C.pSVneo(plasmid control,40X) C'.pSVneo(100X)

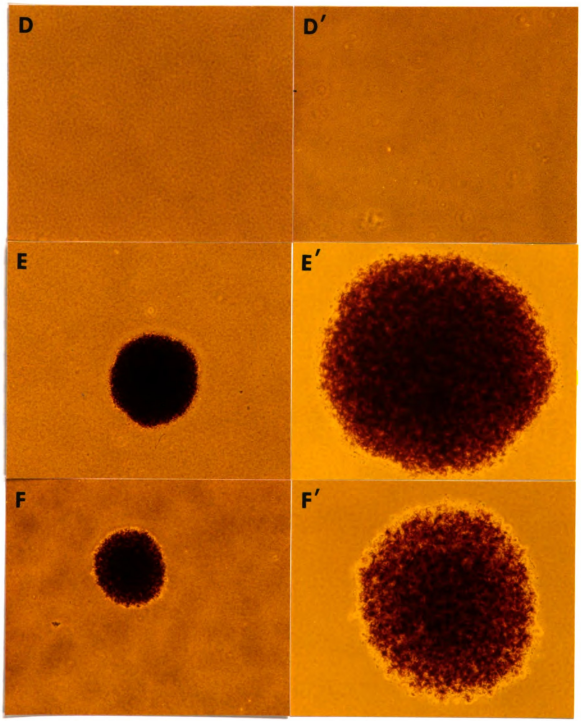

Figure 12(b). AIG of the various cell lines (cont.). D.WB-v-raf A1(40X) D'.WB-v-raf A1(100X) E.WB-v-raf A2(40X) E'.WB-v-raf A2(100X) F.WB-v-raf I(40X) F'.WB-v-raf I(100X).

DISCUSSION

Several results obtained from this research revealed that the integration and expression of v-raf in WB-F344 cells resulted in their neoplastic transformation and tumorigenicity. First, the v-raf transfected clone, WB-v-raf I, had transformed morphology which is spindle-shaped. The cells expressed both v-raf mRNA and raf protein kinase in northern and Western blot analyses respectively. Second, these cells showed the ability of anchorage-independent growth. Such an ability is normally, but not always, indicate of tumorigenicity. Third, the WB-v-raf I cells were found to revert to normal morphology after a few passages and simultaneously lost the v-raf mRNA. In other words, the expression of v-raf mRNA and transformed morphology seems to be reversible and correlated. These results are evidences that v-raf is responsible for neoplastic transformation of the WB-F344 cells. This finding is consistent with earlier observation done by Dr. Worland although the experiment was done with different cell line (Worland et al.1990). Since v-raf is the oncogenic form of cellular raf oncogene, this conclusion is in agreement with the finding that the activation of c-raf proto-oncogene can lead to transformation of rat liver epithelial cells (Heidecker et al. 1990).

It has been suggested that neoplastic transformation and tumorigenicity normally require the cooperation of at least two oncogenes. While this is true for primary cells which require an immortalization step prior to transformation, this proposal may hold true for already immortalized cell lines, since the latter have already escaped differentiation owing to the expression of certain oncogenes, such as c-myc. For example, Trosko et al have reported that WB-cells or RLE cells transfected with v-Ha-ras were neoplastically transformed and were tumorigenic. Familiarly, many laboratories have reported transformation of NIH 3T3 cells upon transfection with a single oncogene. More recently cells transfected with v-raf oncogene were also shown to be neoplastically transformed

(Worland et al 1990). In all of these observation and that of the present study it is probable that all the transformed clones may also express a cooperating oncogene. In many such instances v-myc or c-myc has been implicated as the cooperating oncogene.

In the experiment done by Worland (worland et al. 1990) showed that the level of v-raf expression in those v-raf transduced clones did not directly correlate with the degree of the transformation or tumorigenicity since two v-raf transduced cell lines exhibited marked differences in tumorigenicity despite similar levels of v-raf expression. Therefore, they concluded although v-raf expression is presumably the initiating event in a process that results in transformation of these cells, other unknown events are required for their progression to a highly tumorigenic phenotype. In the present study, similar results were obtained with WB-v-raf transfected cells. Unlike the WB-v-raf I clone, the other clone, WB-v-raf A2, which had the same level of v-raf mRNA expression exhibited normal morphology as parental cells. Anchorage independent growth (AIG) assay indicated that WB-v-raf A2 cells were capable of proliferating in soft agar with fewer numbers of colonies than the WB-v-raf I did. In addition, unlike in the case of WB-v-raf I cells raf protein kinase was not detectable in WB-raf A2 cells in Western blot analyses. These finding indicate that the level of raf protein kinase played significant role in neoplastic transformation. This assumption is supported by the observation that colonies from the soft agar expressed transformed morphology when plated on plastic dishes. However, we do not know if these cells also express the v-raf protein.

In earlier study done by Worland and his associate (Worland et al.1990), it was found that RLE (rat liver epithelial) cells transformed with the v-raf/v-myc combination were capable of anchorage-independent growth in soft agar, while those cells transformed with v-raf alone were not (Worland et al. 1990). In contrast, the WB cells transformed with v-raf alone (WB-v-rafI) can form colonies in soft agar in our study. It was found that the most consistent association between in vitro phenotype and tumorigenesis was the ability of the cells to form colonies in soft agar, so the transformed clone might be

tumorigenic. Carcinogenesis is a complex process by which a normal cell undergoes multistep changes toward transformation. This conversion is of a multistep nature as determined by studies of tumor development and progression both in human, as well as in experimental animals (Pitot et al. 1981). An operational staging assumes three major phases of carcinogenesis namely initiation, promotion and progression (Boutwell 1974, Carins 1975). Thus the immortalized cell line, WB-F344, transfected with v-raf is a good model for tumor progression and it allows in vitro study of genotypic and phenotypic changes accompanying tumor progression.

In this study, it has also been shown that v-raf transfected cells have significantly reduced levels of GJIC as measured by a dye transfer technique. This reduction was more pronouced in WB-v-raf I clone than in the others. Downregulation of GJIC has been implicated as one of the control events contributing to the promotion and progression of tumorigenesis. Many cell lines transformed by oncogenes such as src, ras,neu were found to be unable to communicate either homologously and/or heterologously.

Nicolson et al (1988) have suggested that highly metastatic tumorigenic cells fail to communicate even homologously while less metastatic cells may communicate homologously but not heterologously (with normal cells). Although several of the WB-v-raf clones isolated in the present study still expressed high levels of homologous communication, it is possible that they may not be able to show heterologous communication and therefore can still be tumorigenic. In the current study we also found that the raf transformed cells express CX43 gene similar to the parental cell line.

Another significant observation in the present study was the response of the v-raf transfected clones to the tumor promoting phorbol ester, TPA. Oh et al (1989) reported that in the parental WB-F344 cells, TPA (10 ug/ml) causes only a transient inhibition of GJIC. Communication competence of the TPA treated cells reverts to normal level after 6-8 hrs inspite of the continued presence of TPA. However, these cells were refractory to an additional treatment with TPA and GJIC was unaffected. These authors showed that

the calcium and phospholipid-dependent protein kinase C was involved in the loss of communication among those cells upon TPA-treatment. Presumably gap junction proteins are subtracts for phosphorylation by PKC. It has also been suggested that cells continually exposed to TPA became desensitized to this chemical due to the dissociation (proteolytic cleavage) of PKC from the plasma membrane. In contrast to the transient loss of GJIC in TPA-treated parental WB-cells, in the v-raf transfectant TPA's effect on GJIC was sustained for at least up to 24 hrs. This observation suggests that v-raf transfection may have resulted in the sustained activation of PKC. The mechanism for this change needs to be investigated.

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