EFFECTS OF H-RAS ONCOGENE EXPRESSION ON GAP JUNCTION-MEDIATED INTERCELLULAR COMMUNICATION IN MAMMALIAN CELLS

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ABSTRACT

EFFECTS OF H-RAS ONCOGENE EXPRESSION ON GAP JUNCTION MEDIATED INTERCELLULAR COMMUNICATION IN MAMMALIAN CELLS

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One form of intercellular communication is mediated by the membrane channels known as the gap junctions. These channels allow the exchange of ions, nutrients and regulatory molecules among coupled cells *in vivo* and *in vitro*. This phenomenon of junctional communication has been implicated in the regulation of tissue homeostasis, cell growth and differentiation, as well as synchronization of tissue function and regeneration. Modulators of gap junctions include chemical tumor promoters, growth factors, hormones, neurotransmitters and products of certain oncogenes.

Among a growing number of known oncogenes, members of the ras oncogene family are found expressed in a large number of naturally occurring human malignancies. Recent evidence indicates that the normal or oncogenic ras product, a membrane bound protein, might be an integral component of the inositol phosphate transmembrane signal transduction system. This system was found to be activated by the potent tumor promoter, TPA, and the src oncogene product and correlates with the inhibition of junctional communication.

In order to investigate a possible involvement of the ras product in the modulation of gap junctions, the Chinese hamster V79 cells were transfected with the human c-Ha-ras-1 oncogene. The wild type

cells and those transfected with H-ras were examined for their ability to communicate in vitro. The expression of the ras oncoprotein was detected by indirect immunofluorescence using a monoclonal antibody against p21. Two different techniques were utilized to measure gap junction-mediated intercellular communication. First, a newly developed, rapid and sensitive scrape-loading/dye transfer assay which allows the monitoring of the diffusion of fluorescent dye, presumably across patent gap junctions, in contiguous cells in vitro. The second is the metabolic cooperation assay which measures the transfer of toxic metabolites among coupled cells in culture.

The results obtained from testing H-ras transfected and non-transfected cells indicate significant correlation between the expression of the p21 protein and the blockage of gap junctional communication. The data implicate an important potential role of the ras oncogene in the inhibition of intercellular coupling. This functional similarity between the effect of ras expression and that of certain tumor promoters on gap junction suggests a role the ras oncogene might play in the stage of tumor promotion during the process of carcinogenesis.

To my parents,

To Ann

for their love and support

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INTRODUCTION

The purpose of this study was to investigate a potential role the H-ras oncogene product might play in the modulation of gap junctional intercellular communication in mammalian cells. A suspected resemblance between the properties of the ras oncoprotein and those of some chemical tumor promoters suggested a possible mechanistic correlation between the function of both factors in modulating cellular coupling. In order to conduct this research, the human c-Ha-ras-1 oncogene, derived from the EJ/T_4 bladder carcinoma (Shih and Weinberg 1982), was introduced into communication-competent Chinese hamster fibroblasts. These cells were then tested for their ability to communicate via their gap junctions before and after the introduction, and expression, of the ras oncogene. The expression of the ras oncoprotein was verified by indirect immunofluorescence utilizing monoclonal antibody against v-ras product. The intercellular, gap junction-mediated, communication was tested by two different techniques, the metabolic cooperation and the scrape-loading/dye transfer assays.

This research was undertaken in an attempt to elucidate a potential role for the ras oncogene in the modulation of gap junctional communication following cellular transfection. The transformation of a normal cell to tumorigenic and metastasizing one appears to involve a multistep process of genetic and epigenetic changes (Linder and Gartler 1965, Marchalonis and Nossal 1968, Fialkow 1974, Nowell 1976, Trosko 1987). This neoplastic conversion seems to begin in a single

"initiated" cell (Nowell 1976, Fialkow 1979). According to the concept of initiation, promotion and progression stages of carcinogenesis (Potter 1982), an initiated cell is one that sustained a mutation caused by errors of DNA repair or replication. During the stage of tumor promotion, the initiated cell becomes clonally amplified thus increasing the target size and therefore the chance for further genetic damage (Yotti et al 1979, Trosko and Chang 1984, Potter 1980,1981). The initiated cells then progress toward malignancy and become invasive and metastatic.

Cancer has been regarded to be the result of homeostatic disturbance (Furth 1963, Iversen 1965). It has also been considered as a "stem cell disease" (Till 1982) and a "disease of differentiation" (Markert 1968, Pierce 1974, Potter 1978). Most tumor cells lack contact inhibition when grown in monolayer cultures (Borek and Sachs 1966, Abercrombie 1979). This phenomenon of contact inhibition is believed to play an important role in the control of cell proliferation (Levine et al 1965). Many tumor cells showed absence of gap junctional coupling (Corsaro and Migeon 1977, Loewenstein and Kanno 1966, Fentiman et al 1979, Chang et al 1987). The phenomenon of gap junction-mediated communication represents an important means by which cells can interact directly with each other to equilibrate and exchange ions, nutrients and regulatory molecules of approximately 1000-1500 daltons (Loewenstein 1981). This gating mechanism has been implicated in the regulation of tissue homeostasis, cell proliferation and differentiation (Loewenstein 1979, Bennett and Spray 1985, Pitts and Finbow 1986). The discovery that several tumor promoters, including chemical and physical agents, growth factors and hormones inhibit gap junctional

coupling, provided substantial evidence for an important role gap junctions might play in carcinogenesis (reviewed in Madhukar et al 1988). Recent studies indicate that the products of certain transforming genes, i.e. oncogenes, are themselves growth factors or growth factor receptors (Bradshaw 1986). Some of these oncogene proteins are localized in, or close to, the plasma membrane (e.g. src, ras, erb and neu). The src oncogene was reported to modulate gap junctional communication when expressed in transformed mammalian cells (Azarnia and Loewenstein 1984a, 1984b, Chang et al 1985, Atkinson et al 1986, Azarnia et al 1988). In addition to src, other oncogenic proteins, e.g. the SV40 little and large T antigens (Steinberg and Defendi 1981), and more recently, its middle T antigen (Azarnia and Loewenstein 1987), have been found to correlate with the inhibition of gap junctions. A possible mechanistic link between the expression of certain oncogenes and the inhibition of gap junctions during the process of cancer development was thus entertained (Trosko and Chang 1986, Trosko et al 1983, 1987).

Among the growing number of transforming genes, members of the ras oncogene family are found expressed in a wide variety of naturally occurring human malignancies from different tissues (Willecke and Schafer 1984, Barbacid 1987), as well as in metastatic tumors (Egan 1987, Collard et al 1987). The ras oncogene differ from the normal proto-oncogene by a single point mutation and the mutated ras product has decreased GTPase activity as compared to the wild type (reviewed in Barbacid 1987). Ras protein is also known to modulate the adenylate cyclase signaling system in cells expressing p21 (Hiwasa and Sakiyama 1986). Although the function of the normal or oncogenic ras protein is not fully understood, recent evidence indicate that it represents an

integral component of the phosphatidylinositol 4,5 diphosphate (PIP2) membrane signal transduction system (Fleishman et al 1986, Wolfman and Macara 1987). Interestingly, this is the same membrane system that might be activated by the src oncogene product (Chang et al 1985) and by TPA, the potent tumor promoter, 12-O-tetradecanoylphorbol-13-acetate (Castagna et al 1982). The signal transduction through the PIP2 system leads to the activation of a calcium sensitive protein kinase C (PKC) receptor/enzyme (Nishizuka 1986). PKC activation has been correlated with the inhibition of gap junctional intercellular communication (Enomoto and Yamasaki 1985b, Castagna et al 1982). In addition, an observed synergism between TPA and the ras oncogene during the process of *in vitro* transformation might suggest a common biological function for both TPA and the ras protein p21 (Dotto et al 1985). Therefore, in order to explore the possible functional correlation (or the lack of it) between the expression of the ras p21 and intercellular communication, the ability of the mammalian Chinese hamster V79 fibroblasts to perform gap junction coupling was tested before and after the introduction of the mutant, oncogenic human c-Ha-ras-1 gene. The expression of p21 protein was verified by indirect immunofluorescence using a monoclonal antibody against V-ras p21, and the gap junction-mediated intercellular communication assayed by the two different techniques, the metabolic cooperation and the scrape-loading/dye transfer.

LITERATURE REVIEW

Cell-to-Cell Communication

Intercellular communication in multicellular organisms has been recognized as an important determinant in the regulation of homeostasis, growth, development, cell proliferation and differentiation (Saxen et al 1976, Loewenstein 1968, Sheridan 1976, Blumberg et al 1982, Wolpert 1978, Bennett et al 1981, Gilula 1980 and Yamasaki 1984). Cellular interactions can be fulfilled by two major mechanisms. In the first, the cells transmit signals across the extracellular space as is the case with growth factors, hormones, neurotransmitters, and other mediators, where the target cells with appropriate receptors could be located in a distant tissue or organ. The other form of cellular interactions, on which the emphasis of this dissertation will be focused, involves cells in close contact. These cells communicate via specialized structures called the "gap junctions" (Loewenstein 1981).

This form of cellular communication was first observed as an electrical synapse in the invertebrate nervous system (Furshpan and Potter 1959, Bennett 1977). Following that initial observation many metazoan tissues were found to have similar modes of membrane conductance e.g., in coelenterates (Hand and Gobel 1972, Wood and Kuda 1980), in fish (Robertson 1963) and in mammals (Revel and Karnovsky 1967). The mammalian myocardial cells showed remarkable reliance on junctional communication in the synchronization of propagating the action potentials (Weidmann 1952, Dreifuss et al 1966).

Cells coupled via gap junctions form a large cytoplasmic syncytium in which relatively small size organic molecules and ions can diffuse freely among contacting cells (Hertzberg et al 1981, Loewenstein 1981, Spray et al 1982). These gap junctions are found in almost all metazoan organisms and they seem to have been highly conserved throughout evolution (Perrachia 1973). The general structure and functional properties of arthropod and vertebrate gap junctions (Simpson et al 1977, Finbow and Pitts 1981), as well as those in a wide range of tissues of multicellular organisms are much the same (Peracchia 1980). Studies with antibodies derived against liver gap junction protein have detected homologous polypeptides in different tissues (Dermietzel et al 1984, Hertzberg and Skibbens 1984). In addition, in vitro studies indicate that gap junction coupling can take place between cells from different metazoan organs and organisms (Epstein and Gilula 1977, Michalke and Loewenstein 1971, Gaunt and Subak-Sharpe 1979, Flagg-Newton and Loewenstein 1980).

The models suggested by Sheridan (1976) and Loewenstein (1979) describe the effect of cell coupling on the concentration of hypothetical diffusible molecules that regulate cell division and differentiation. The concentration of such molecules could be dependent on their rate of synthesis and on the total volume of cytoplasmic syncytium in which they are distributed. A physiological equilibrium level could be disturbed by the interference with the gap junctional communication. Such inhibition or down regulation of gap junction function could be triggered by multitude of factors. Uncoupling could occur after terminal differentiation, cell death, physical exclusion, mitogenic signals, altered ionic concentration, endogenous or exogenous factors or

chemicals to be described in the following sections. The consequence of such disturbance of this cellular function could potentially vary from no noticeable effect to tumor promotion by permitting the proliferation of initiated cells; organ dysfunction, e.g., in the myocardium, causing abnormal signal transduction; or disease conditions by disturbing organ or tissue homeostasis (Trosko and Chang 1980).

Gap Junction Structure

In 1972, Gilula and colleagues demonstrated the presence of a specialized membrane structure that is responsible for the transfer of small molecules and electrical signals between contacting cells (Gilula et al 1972). Extensive biochemical, electron microscopic and x-ray diffraction studies have been conducted to study gap junction structure in different tissues from different organisms and at different stages of development and differentiation (for review see Peracchia 1980).

Gap junctions are specialized transmembrane structures that are composed of multiple channels or connexons. Each connexon is composed of six identical rod-shaped polypeptide subunits of about 7-8 nm long arranged as oligomers that form continuous channels between two adjacent cytoplasms allowing for molecule transfer. Each connexon protrudes for about 2 nm in the extracellular space to join its counterpart from a contiguous cell thus forming a "gap" seen interrupting the closely apposed cell membranes when stained and examined by electron microscopy (Revel and Karnovsky 1967). The gap junction units tend to aggregate in plaque-like structures that vary in size, shape and distribution in various cells from different tissues. The apposed junctions from two adjacent cells align together to form continuous aqueous

channels that provide direct communication between their cytoplasms. These pathways allow the free diffusion of relatively small molecules and ions of 1000-1500 daltons MW (Loewenstein 1981) between coupled cells (Simpson et al 1977). Each cell is able to maintain its individual characteristics by retaining specific non diffusible macromolecules (Gilula 1985).

The gap junction protein has been isolated from rodent liver cells (Revel et al 1985). A protein of a molecular weight of about 27 K daltons has been identified (Hertzberg and Gilula 1979, Henderson et al 1979, Hertzberg et al 1981). Polyclonal antibodies prepared against liver gap junction proteins were able to recognize similar antigenic determinants in a wide variety of tissues from different species (Hertzberg & Skibbens 1984, Hertzberg 1980, Ziegler and Horwitz 1981, Traub et al 1982, Willecke et al 1985). This similarity suggests an evolutionary conservation of commonly recognizable antigenic determinants of the gap junction proteins in different organisms and tissues and, therefore, underlines the junctional importance in multicellular organisms.

Gap Junctions and Development

The role of membrane gap junctions during development has been extensively studied (Bennett 1973) and considered to control the diffusion of second messengers that regulate cell proliferation and differentiation (Lawrence et al 1978). Prior to fertilization and during the process of oocyte maturation in a variety of organisms (e.g., mice, sheep, chickens and frogs), it was discovered that cells of the cumulus granulosa (members of follicular cell population) do establish direct

communication with oocyte through processes that penetrate the layer of the zona pellucida (reviewed in: Schultz 1985, Gilula 1978). Ovulation appears to be triggered by hormonal stimuli with subsequent disruption of this form of cell-cell communication. Following fertilization, studies of electrical and dye coupling in mouse embryos showed that intercellular communication begins among all embryonic cells at the eight cell blastomere stage and throughout the blastocyst formation. Further development and differentiation was found to correlate with compartmentalization of cell coupling (reviewed in Gilula 1980, Spray et al 1982, Warner 1983, Schultz 1985). In Drosophila, the pattern of intercellular coupling was studied in the embryonic wing imaginal disc during its development (Lo 1985). Several developmental compartments showed restricted pattern of cell coupling. Similar observations were made after studying junctional communication in various insect epidermis (Warner and Lawrence 1982, Blennerhassett and Caveney 1984). In addition to its potential role in embryo development, gap junctional intercellular communication is thought to participate in the regulation of cellular proliferation and differentiation in the fully developed organism (Loewenstein 1979, 1981, Gilula 1985, Lo 1985, MacDonald 1985, Neyton and Trautmann 1986, Pitts and Finbow 1986, Revel et al 1985, Schultz 1985). These findings strongly point to functional correlation between gap junctional communication and tissue development, organization and differentiation.

Direct evidence of the role of gap junction intercellular communication in development and differentiation was demonstrated when antibodies against gap junction proteins were microinjected in cells from developing embryos (Warner et al 1984, Hertzberg et al 1985). Gap

junctional intercellular communication was blocked or reduced in Xenopus laevis embryo cells after microinjection of antibodies against the gap junction proteins. More than half the embryos that were allowed to mature developed congenital patterning anomalies corresponding to the segments that were injected with antibodies (Warner et al 1984).

Distribution of Junctional Communication

The study of the patterns of intercellular communication in intact tissues and organs suggest a role for gap junctions in the control of growth and differentiation. Gap junctional intercellular communication was found to have variable distributions in intact differentiated organs and in different types of epithelial tissue. The results indicated that most epithelial cells are electrically coupled e.g. in pancreas, lacrimal and salivary glands (Petersen 1980). In mammalian salivary glands, gap junction communication exists between cells within the same ascini but not between ascini of the same gland (Petersen 1980). In the testis, sperm maturation appears to depend on the presence of intact gap junctions between the Leydig and Sertoli cells (Sharpe et al 1981).

Similar pattern of communication was detected among hepatocytes of weanling rats by electric coupling and fluorescent dye transfer (Meyer et al 1981). The extent of this permeability was downregulated following partial hepatectomy and the start of organ regeneration. Microinjection of Lucifer yellow dye in intact adult mouse skin showed extensive dye transfer among dermal fibroblasts and a limited diffusion in epidermal keratinocytes (Pitts et al 1987). There was no dye transfer across the basement membrane. The dermal fibroblasts were not coupled to the neighboring cells of the sebaceous glands or the muscle fibers

(Pitts et al 1987). In the same study, the cells in sebaceous glands were totally coupled among themselves yet completely uncoupled with dermal or other contiguous cells. These observations indicate that intercellular permeability could be selective among cells of the same organ or tissue most likely to maintain functional, biochemical and/or electrical syncytia.

Modulation of gap Junction Function

An important observation linking tumor promoting agents to the inhibition of gap junctional communication was made by Yotti et al (1979) and Murray and Fitzgerald (1979). The potent tumor promoter phorbol ester TPA (12-O-tetradecanoylphorbol-13-acetate) was found to inhibit the metabolic cooperation between cells grown in culture. This observation was further substantiated in various cell types using different tumor promoters (Enomoto et al 1981, Trosko et al, 1982, Kalimi and Sirsat 1984, Enomoto and Yamasaki 1985, Yancey et al 1987, Trosko et al 1987, Tsushimoto et al 1983, Warngard et al 1985). Different methods to measure gap junction communication were utilized to test the effects of various chemical compounds on the membrane channels e.g. dye transfer by microinjection (Enomoto and Yamasaki 1985b), electrocoupling (Enomoto et al 1981), FRAP analysis (Wade et al 1986) and scrape-loading/dye transfer (El-Fouly et al 1987).

Using freeze-fracture analysis, cells treated with tumor promoters were found to contain less gap junctions, when compared to control *in vitro* (Yancey et al 1982) and *in vivo* (Kalimi and Sirsat 1984). The tumor promoter, phorbol ester, was also found to enhance cellular transformation while blocking intercellular communication (Enomoto and

Yamasaki 1985b). TPA is known to activate a membrane-bound, calcium-sensitive receptor enzyme protein kinase-C (PKC) (Castagna et al 1982). A strong correlation between the activation of PKC and the inhibition of gap junctions has been therefore implicated. Recently it was shown that activated PKC phosphorylates gap junction proteins of rat liver cells in a cell-free system (Takeda et al 1987). PKC is also known to be activated by an endogenous second messenger, diacylglycerol (DAG), a byproduct of phosphatidylinositol 4,5 biphosphate (PIP2) (Kishimoto et al 1980, Nishizuka 1984, Enomoto and Yamasaki 1985, Gainer and Murray 1985). This PIP2 is a major component in a transmembrane signaling system that transmits external signals into the cell to provoke biological response. Another byproduct of PIP2 breakdown is the inositol triphosphate which releases the intracellular calcium from its stores in the endoplasmic reticulum. This release raises the cytosolic concentration of ionic calcium (Berridge and Irvine 1984), which consequently activates PKC (May et al 1985) and inhibits the junctional communication (Rose and Loewenstein 1977, Rose and Rick 1978).

Other endogenous factors that are known to modulate gap junctional function include the intracellular pH (Turni and Warner 1977, 1980, Spray et al 1981) and cyclic AMP (Azarnia et al 1981, DeMaziere and Scheuermann 1985, Flagg-Newton et al 1981, Kanno et al 1983, Mehta et al 1986, Saez et al 1986). Increased levels of cAMP appear to enhance junctional communication among contiguous cells (Saez et al 1986, Wiener and Loewenstein 1983, Johnson et al 1985). Recently, certain membrane bound proteins, e.g. the src oncogene product, have been shown to be associated with the modulation of intercellular coupling (Azarnia and Loewenstein 1984a,b, Chang et al 1985, Atkinson and Sheridan 1986).

Furthermore, changes in the membrane potential (Spray et al 1979, Harris et al 1983) or alteration of the adhesion molecules that assist in maintaining close juxtaposition of contiguous cells (Edelman 1983, Obrink 1986) could also modify the cells' ability to conduct intercellular communication.

Extracellular or "exogenous" factors, including tumor promoting agents, were found capable of modulating the junctional gating system. Alcohols (Johnston et al 1980), hormones (Merk et al 1972, Decker 1976, 1981, Dahl and Berger 1978, Garfield et al 1980), neurotransmitters e.g., acetylcholine (Neyton and Trautmann 1986), vitamin A and its derivatives (Elias and Friend 1976, Elias et al 1981), certain drugs e.g., phenobarbital (Jone et al 1985), valium (Trosko and Horrobin 1980), dietary elements e.g., unsaturated fatty acids (Aylsworth et al 1984) and saccharin (Trosko et al 1980), environmental pollutants e.g. polybrominated biphenyls (Tsushimoto et al 1982), solvents (Chen et al 1984), metabolites (Malcolm et al 1985), phorbol esters (Murray and Fitzgerald 1979, Yotti et al 1979, Fitzgerald and Murray 1980, Enomoto et al 1981, Trosko et al 1982, Kalimi and Sirsat 1984, Enomoto and Yamasaki 1985, Yancey et al 1987), dieldrin (Trosko et al 1987), DDT (Tsushimoto et al 1983, Warngard et al 1985), growth factors e.g., EGF and TGF-β (Madhukar et al 1988) were all shown capable of modulating gap junction function.

Physical factors e.g., localized cell death or surgical resection in organs as in partial hepatectomy, induce cell proliferation during the regeneration and healing processes and are associated with the reduction of gap junction number and, consequently, with the inhibition of cellular coupling (Yee and Revel 1978, Yancey et al 1979, Meyer et

al 1981). Other gap junction function modifiers include factors like CO_2 concentration (Turin and Warner 1977) and changes in temperature (Arancia et al 1986).

The multitude of factors and different classes of chemicals and physical agents that share an ability for modulating gap junction function might be operating via different mechanisms yet through a few cellular signals or key regulatory elements e.g. pH, cAMP or Ca^{++} concentration, to induce cellular response i.e. proliferation or differentiation. In addition to the known factors that modulate gap junctional intercellular communication, one might speculate that at the molecular and biochemical level, mutations affecting the gap junction gene or any of its regulatory elements or other genes regulating gap junction function, or any alterations of their expression, translation, post translation modification or membrane translocation, orientation or internalization might, as a consequence, affect the junction-mediated communication. Depending on the nature of the junction modulator, exposure time, stage of cell cycle, state of differentiation and/or proliferation, the cellular response could be adaptive or maladaptive (Trosko and Chang 1984). In the latter situation the consequences could vary from unremarkable to disruptive of normal function and or proliferation (Trosko and Chang 1980, Trosko et al 1987).

Assays for Measurement of Gap Junctional Communication

Given the passive nature of gap junctional permeability, it appears that the control of signal transmission between contiguous cells might depend on different factors. Among these, the number of available membrane junctions, their diameter, the concentration gradient of the

diffusible molecules and their variable friction factors. A number of assays have been devised to measure the junctional permeability between cells either directly or indirectly. The following is a brief description of those available methods that are being successfully used to study gap junction functions. Some of these assays are reviewed in more detail elsewhere (e.g., Socolar and Loewenstein 1978).

1. Electrical coupling: In this assay, microelectrodes are placed intracellularly to measure electric (ionic) currents between contacting cells and between cells and the extracellular medium (Azarnia and Loewenstein 1971, Enomoto et al 1981, Yamasaki et al 1983). It is a sensitive assay that can be applied in vitro and in vivo to cells of as small a diameter as 10 um (Loewenstein 1979). It is not capable however of testing for transfer of molecules larger than the small charged ions it detects.
2. Junctional electric conductance: This method allows the quantitation of electrical coupling. It is a complicated procedure that requires several microelectrodes and compares various parameters including nonjunctional membrane resistance or cellular input resistance. Its application may be useful in simple cell systems (Socolar and Loewenstein 1978).
3. Transfer of radiolabelled molecules: This method monitors the transfer of tritiated uridine nucleotides from donor to recipient cells grown in co-culture. The recipient cells can be easily identified by prelabeling with fluorescent microspheres before the co-culture (Keijzer et al 1982). The degree of cell-cell communication via gap junctions can be estimated by scoring for presence

of transferred radiolabelled molecules (Murray and Fitzgerald 1979). This method is lengthy and somewhat tedious and the results are obtained after about 2-3 weeks from start of experiment.

4. Metabolic cooperations assays: These assays rely on the fact that cells in contact are capable of exchanging a number of metabolites among themselves across gap junctions (Subak-Sharpe et al 1966, Burk et al 1968). Mutant cells with deficiency in the pyrimidine or purine synthetic pathways can survive in selective media if co-cultured and contacted with wild type cells (Cox et al 1970, Pitts 1971, Pitts and Simms 1977). The transfer of molecules is presumed to occur via gap junctions since studies have indicated strong correlation between metabolic cooperation and electrical coupling (Gilula et al 1972, Azarnia et al 1972). Cell coupling can be measured by transfer of radioactive metabolites (Subak-Sharpe et al 1968), or by assessment of the number of surviving colonies of HPGRT-deficient mutants when co-cultured with wild type cells in the presence of 6-thioguanine, 6TG (Fujimoto et al 1971, Corsaro and Migeon 1977).

   In the latter modification of the assay the wild type cells would metabolize the 6TG to a toxic phosphorylated byproduct which results in cell death. The mutant HGPRT-deficient cells are inherently resistant to the lethal effect of 6TG since they are incapable of metabolizing it. When both cell types are grown in co-culture in the presence of 6TG, the toxic metabolite 6-thioguanine monophosphate can thus be transferred between contacting cells with competent junctional communication and is capable of killing the mutant cells. On the other hand, if the gap junctions are

totally or partially inhibited, the mutant cells survive to form colonies which can be easily scored and compared to the plating efficiency of similar number of mutant cells.

The metabolic cooperation assay allows the testing of chemicals exerting long term modulation of gap junctions at non cytotoxic doses and also provides a good means for quantitative analysis of dose-response effects of potential modulators of gap junction communication. The use of this method however is limited to a few established mammalian cell systems. The cells should have a relatively high colony forming ability which is not the case in primary cells. The assay also requires the utilization of well characterized mutant cells and it does not allow for testing of short term, or reversible, inhibition of gap junction.

5. Fluorescent dye transfer: The intracellular microinjection of membrane impermeable tracer molecules e.g. Lucifer yellow (LY) has been successfully utilized to study gap junctional permeability in coupled cells (Rose et al 1977, Flagg-Newton and Loewenstein 1979, Flagg-Newton et al 1979, Friedman and Steinberg, 1982). This method allows the direct visualization of intercellular coupling. It also assists in the quantitative evaluation of the degree of junctional competence by counting the number of secondary dye recipient cells and is applicable to a wide variety of cells from different tissues, organs and species. It is a sensitive assay and can be utilized to detect the permeability limits of molecular size (Azarnia and Loewenstein 1976, Rose et al 1977).

6. Fluorescence Recovery After Photobleaching (FRAP) analysis: This is a technologically advanced extension of studying gap junction

communication using fluorescent tracer molecules (Wade et al 1986). The fluorescent dye 6-carboxyfluorescein diacetate (a hydrophobic membrane-permeable nonpolar ester) is absorbed by cells grown in culture. Once in the cytoplasm it becomes rapidly hydrolyzed by esterases yielding free fluorescein, a molecule with small enough size to permeate across open gap junctions between coupled cells. The procedure involves laser photobleaching of single cells, coupled and noncoupled, and assessing the degree of dye redistribution across gap junctions from contiguous non-photobleached cells. When contacting cells are pretreated with chemicals known to inhibit gap junction communication, no dye recovery is observed in the photobleached cells. This assay is very sensitive yet requires sophisticated equipment and computer processing to analyze and quantitate the data.

Inhibition of Gap Junctional Communication and Tumor Promotion

Carcinogenesis is a complex process by which a normal stem cell undergoes multiple changes toward transformation. This conversion is of a multistep nature as determined by studies of tumor development and progression both in humans, as well as in experimental animals (Foulds 1954, Nowell 1976, Cairns 1975,1981). The clonal origin of cancerous tumors has been determined in several studies (Fialkow 1976, Nowell 1976, Baylin et al 1978). An operational staging assumes three major phases of carcinogenesis namely initiation, promotion and progression (Boutwell 1974, Pitot et al 1981, Foulds 1954, Cairns 1975, Berenblum and Armuth 1981).

During the initiation phase, a stem cell sustains a stable and

irreversible "premalignant" genetic damage which could be inherited or induced by a given mutagen (Ames et al 1973). Mutation fixation is achieved by an error-prone repair or replication of DNA (Glover et al 1978, Maher and McCormick 1977, Warren et al 1981). The majority of mutagens have been shown to play a role in initiation or sometimes behave as "complete" carcinogens (Trosko et al 1983). An initiator, at a high enough and cytotoxic dose, might cause extensive damage to the cells following exposure and might lead to cell killing. This killing could provide promoting conditions by inducing the surviving cells, including those initiated stem cells that escaped death, to proliferate (Loch-Caruso and Trosko 1985). There is strong evidence to implicate mutagenesis in carcinogenesis (Trosko and Chang 1981, Yuspa and Morgan 1981), yet carcinogenesis, as a complicated process, requires more than just mutations (Trosko and Chang 1978, Trosko et al 1985).

In the promotion phase of carcinogenesis, selective proliferation, i.e. clonal amplification, of the initiated cells by promoters or mitogens takes place (Trosko and Chang 1983, Trosko et al 1983). The initiated stem cells have a natural growth advantage over non-stem cells given their ability for self renewal and lack of differentiation (Chang et al 1987, Bykorez and Ivashchenko 1984, Kondo 1983, Steel and Stephens 1983, Till 1982). The proliferation of the initiated, premalignant cells to reach a "critical mass", increases their chance of sustaining additional mutational events and thus enhancing the probability of tumor formation (Boutwell 1974). During this stage of carcinogenesis, i.e. the stage of tumor progression, the cells acquire a series of morphologic, biochemical and genetic changes that would, in most cases, alter the normal differentiation pathway (Sachs 1980 a,b),

lead to rapid, autonomous growth, invasion and metastasis (reviewed in Trosko and Chang 1984a,b).

In the absence of tumor promotion, the initiated cells presumably remain quiescent through a homeostatic control contributed by their normal counterparts. This condition appears to be in contrast with cancer cells which are thought of as cells that lost homeostatic control (Furth 1963, Iversen 1965) and the capacity for differentiation (Potter 1978). This assumption was strengthened by the fact that most malignant cells lack contact inhibition (Borek and Sachs 1966, Corsaro and Migeon 1977), and the majority have lost the ability to communicate with normal cells via gap junctions (Loewenstein 1979, Loewenstein and Kanno 1964, Fentiman et al 1979, Kanno 1985). 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, Murray and Fitzgerald 1979). This link was further substantiated when a number of laboratories using different assays to study the effects of known tumor promoters on gap junction permeability in cells from different tissues and organs came to the same conclusions (reviewed in Trosko and Chang 1984a,b, 1987).

Treatment with tumor promoters remarkably decreased the number of gap junctions in V79 cells when compared to the untreated control as revealed by freeze-fracture and electron microscopy studies (Yancey et al 1982). Similar results were obtained in phorbol ester treated mouse epidermis cells (Kalimi and Sirsat 1984 a,b) and in regenerating liver cells in partially hepatectomized animals (Yancey et al 1979, Yee and

Revel 1979). Substantial evidence indicate that tumor promoters act primarily on cell membrane while initiators, acting as mutagens, target the DNA (reviewed in Trosko and Chang 1984a,b). These observations lend strength to the hypothesis that tumor promoters act by blocking gap junctional coupling between normal and initiated stem cells thus disrupting their contact inhibition (Abercrombie 1979, Borek and Sachs 1966) and homeostatic regulation of proliferation and differentiation (Trosko and Chang 1984a,b).

The Oncogene Hypothesis and Cancer

Most tumor cells are believed to have sustained some genetic alterations due to errors in DNA repair or replication (Strong 1977, Cleaver et al 1975). Among the many theories dealing with the origin of cancer, the oncogene concept is gaining wide acceptance (Huebner and Todaro 1969, Todaro and Huebner 1972, Marshall 1986). Oncogenes are genes capable of inducing cellular changes that lead to neoplastic transformation. In the viral hypothesis of oncogenesis, it is postulated that all vertebrate cells contain genes (called proto-oncogenes) analogous to the transforming elements present in tumor viruses (Huebner and Todaro 1969, Todaro and Huebner 1972, Bishop 1983). These proto-oncogenes have been evolutionary conserved in all metazoan organisms. This fact may underline their critical importance in normal cellular function and proliferation. They can acquire oncogenic activities by one or more of four basic mechanisms, namely: inappropriate expression during the wrong stage of cell growth or differentiation, amplification, translocation and mutations. The mutational changes can be induced by radiation, mutagens or viruses. Nonmutational genetic

mechanisms can also play a role in oncogenic activation e.g. by changes in DNA methylation, amplification or epigenetic alteration of proto-oncogene expression (reviewed in Pimentel 1986). The currently identified oncogenes represent a set of highly conserved genes that presumably play an important role in cell proliferation and differentiation (Lacey 1986, Pimentel 1986).

Oncogenes have been classified according to different criteria, e.g., intracellular localization of oncoprotein, biochemical characteristics, or specific structure or function. Two major classes of oncogenes have been recognized based on the localization of their products. These are the nuclear and the cytoplasmic or membrane-bound oncogenes (Weinberg 1985). Several reports have indicated that these two classes of oncogenes can and do cooperate to transform normal primary cells in culture e.g., the nuclear oncogene product of c-myc and either of the membrane-bound Ha-ras or N-ras (Land et al 1983); or N-myc with the activated Ha-ras (Yancopoulos et al 1985). Another classification segregates most of the known oncogenes according to their biochemical activity into two major families (reviewed in Pimentel 1986). First, the src family which includes those oncogenes with tyrosine kinase activity, e.g., src, abl, fms, yes, erb-B, fes, and ros. Second, a group with no known protein kinase activity and this includes myc, myb, mos, sis, and ras oncogenes. The ras family is the most ubiquitous in malignant tumors of human origin (Barbacid 1986). It has three highly homologous members, H-ras, K-ras, and N-ras, all of which bind guanine neucloetides and have been implicated, as discussed below in more details, in transmembrane signal transduction. Certain oncogene products showed remarkable homology to known cellular proteins

(reviewed in Pimentel 1986), e.g., sis is homologous to PDGF, erb-A to carbonic anhydrase, erb-B to EGF receptor, mos to EGF precursor, ras to G-proteins, fes/fgr to actin, myc to beta and gamma crystallins and fms to the mononuclear-phagocyte colony stimulating factor (CSF-1). Some of those proteins are localized at or near the cell membrane, and were thought to have potential functional association with the promotion phase of carcinogenesis given their ability, once activated constitutively or by induction, to act as mitogen and induce cell proliferation (Trosko et al 1983,1987). This hypothetical association also attempts to link the expression of certain oncogenes to the interference with gap junction communication (Trosko and Chang 1986).

Oncogenes and Cell-Cell Communication

Recently, the alteration of gap junctional communication has been described in cells transformed with v-src oncogene (Chang et al 1985, Azarnia and Loewenstein 1984a, 1984b, Atkinson et al 1986). The expression of src protein ($pp60^{src}$), a tyrosine kinase linked to the phosphatidylinositol 4,5 biphosphate (PIP2) transmembrane signaling system (Macara et al 1984, Sugimoto et al 1984), was correlated with increased protein Kinase C (PKC) activation and inhibition of gap junction intercellular communication (Chang et al 1985). Other viral oncogenic products, e.g. the little T and the large T antigens of SV40 (Steinberg and Defendi 1981), and more recently the middle T of the same virus (Azarnia and Loewenstein 1987) have been associated with the reduction of junctional permeability. These findings might reflect a common role played by chemical promoters, oncogenes and viral agents in the inhibition of gap junctions during the process of tumor promotion

(Trosko and Chang 1986,1987, Trosko et al 1984, Madhukar et al 1988).

The Ras Oncogenes

One of the highly conserved and ubiquitous membrane-bound oncoproteins is the p21 polypeptide of the small family of ras oncogenes (Barbacid 1987). These transforming genes were first discovered in the Harvey and Kirsten strains of rat sarcoma retroviruses (Harvey 1964, Kirsten and Mayer 1967). The cellular counterparts of the ras genes (i.e. c-ras) appear to be highly conserved and are found in organisms from yeasts to mammals (Shilo and Weinberg 1981). This may reflect an important role for the ras genes in basic cellular function. Human cells have three functional ras genes namely, c-Ha-ras-1, c-K-ras-2 and c-N-ras which map to chromosomes 11p15.1-p15.5, 12p12.1-pter and 1p22-p32 respectively.

At the molecular level, all ras genes have four exons (with the exception of c-K-ras-2 which has two alternative fourth exons), a 5' non-coding exon and promoter regions containing G/C rich boxes without TATA or CAT boxes, a characteristic of promoters of housekeeping genes (Barbacid 1987). Biochemically, the p21 protein contains four domains: a highly conserved, 85 amino acid first domain, a second domain with 80 amino acids which have 85% homology in the human ras genes, a third with 20 amino acids that show remarkable variability, and last, a conserved stretch of four amino acids including cysteine at position 186 followed by two aliphatic and one variable amino acid (Barbacid 1987). The p21 proteins are found at the cytoplasmic surface of the cell plasma membrane (Willingham et al 1980, Willumsen et al 1984, Fujiyama and Tamanoi 1986). They bind guanine nucleotides (GTP and GDP) (Scolnick

et al 1979, Shih et al 1980, Tamanoi et al 1984, Temeles et al 1985) and have GTPase activity (Temeles et al 1985, Sweet et al 1984, Manne et al 1985).

Ras proteins also appear to be involved in transmembrane signal transduction (Willingham et al 1980, Furth et al 1982, Levinson 1986). The N-terminal domain of the ras gene product (p21 protein) shows homology to the mammalian G-proteins (Hurley et al 1984) which mediate ligand-induced activation of adenylate cyclase and phospholipase C (Wakeman et al 1986). The former controls the cellular cyclic AMP (cAMP) level (Rodbell 1980) and the latter catalyzes the breakdown of phosphatidylinositol 4,5 diphosphate into inositol triphosphate and diacylglycerol (Nishizuka 1986). In the NIH3T3 cells, the ras oncogene expression was found to correlate with decreased activity of the adenylate cyclase membrane signaling system and with low levels of cAMP (Hiwasa and Sakiyama 1986). Decreased cAMP levels correlates with the inhibition of junctional intercellular communication (Saez et al 1986, Wiener and Loewenstein 1983, Johnson et al 1985). In addition,emerging evidence indicates that the p21 protein may be an integral component of the polyphosphoinositide signaling system (Fleishman et al 1986). The expression of either the normal or the oncogenic forms of H-ras in mammalian cells was found to induce altered levels of phosphatidylinositol 4,5 biphosphate (PIP2) and its catabolites (Fleishman et al 1986). One of the PIP2 catabolites is diacylglycerol (DAG). The elevation of DAG levels is known to activate a calcium sensitive, phospholipid dependent protein Kinase C (PKC) (Kishimoto et al 1980, Nishizuka 1984, Enomoto and Yamasaki 1985) and to inhibit gap junctional coupling in cultured cells (Gainer and Murray 1985). This PKC

activation is thought to be a critical step in cell transformation by H-ras (Fleishman et al 1986, Nishizuka 1984, Wolfman and Macara 1987). Interestingly, the potent tumor promoter, 12-O-tetradecanoylphorbol-13 acetate (TPA), directly activates PKC by substituting for DAG (Castagna et al 1982, Niedel et al 1983, Nishizuka 1986) and, like other chemical tumor promoters, it blocks gap junction-mediated intercellular communication (Yotti et al 1979, Enomoto et al 1981, Yancey et al 1987, Kalimi and Sirsat 1984, Trosko et al 1982, Enomoto and Yamasaki 1985, Fitzgerald and Murray 1980).

In addition, it has been recently reported that TPA and H-ras oncogene cooperate in inducing transformation in cells in culture (Dotto et al 1985). This observation suggests a common cellular effect of TPA and the ras gene product. Furthermore, the ras oncogene is reported to confer transformed phenotypes on initiated cells or cells immortalized by myc oncogene, p53, E1a gene or the large T antigen of the SV40 virus (Land et al 1983a, 1983b, 1986, Parada et al 1984, Van Roy et al 1986, Beer et al 1986, Yancopoulous et al 1985, Ruley 1983, Segana and Yamaguchi 1987, Connan et al 1985), as does TPA in cells immortalized by myc oncogene (Connan et al 1985). From the aforementioned observations, it appears that a functional correspondence and a common underlying mechanism might exist between the ras p21 and the tumor promoter TPA. An attractive and testable hypothesis thus arises which proposes that a potential role might be played by the ras oncogene in the process of tumor promotion during the neoplastic transformation. This role of the ras p21 might interfere, directly or indirectly, with the homeostasis and the proliferation ability of the initiated cells by disrupting their junctional coupling. If this were the case, the ras

oncogene might thus be mimicking the actions exerted by chemical tumor promoting agents by being an "endogenous", persistently expressed promoter.

In order to test for potential role of the ras oncogene in the modulation of gap junctional function, the Chinese hamster V79 lung fibroblasts were transfected with the human c-Ha-ras-1 derived from the EJ/T_4 bladder carcinoma. These V79 cells were tested for their ability to communicate in culture before and after transfection with the H-ras oncogene.

MATERIALS AND METHODS

Additional materials and methods are included in the two publications enclosed on pages 61-79.

Cells

Chinese hamster lung fibroblasts (V79 cells) are known to have competent gap junction communication. This fact has been verified by several methods including the metabolic cooperation (Yotti et al 1979), uridine transfer (Trosko et al 1982), and microinjection of fluorescent, membrane-impermeable tracer molecules (Mazzoleni et al 1985, Zeilmaker and Yamasaki 1986). Their gap junctions have also been visualized by freeze-fracture and electron microscopy studies (Yancey et al 1982, Tsao et al 1984). These mammalian cells are also relatively easy to transfect (Liu and Loeb 1984).

Medium and Culture Conditions

Cells were grown in a modified Eagle's medium with Earle's balanced salt solution with a 50 percent increase of essential amino acids and vitamins supplemented with a 100 percent increase of non-essential amino acids, 1 mM sodium pyruvate, and 5 percent fetal calf serum. All cells were incubated at 37^{o}C in water-jacketed incubator with humidified air and 5 percent CO_2.

Plasmids

1. pSV2neo is a recombinant plasmid that contains the aminoglycoside phosphotransferase G418 resistance gene included as a marker to select for transfected cells (Southern and Berg, 1982).
2. $pSWO_4$ was obtained from Dr. S. Warren (Emory University, Atlanta, GA). This is a pSV_2neo in which a 6.6 Kb fragment containing the entire human-c-Ha-ras-1 oncogene from EJ/T_4 bladder carcinoma line, is inserted into the Bam H 1 site (Figure 1).

DNA Transfection Assay

Plasmids were propagated in E. Coli., strain HB101, by the calcium chloride procedure as described in Maniatis et al (1982). V79 cells were transfected by plasmid DNA according to the calcium phosphate precipitation method as described by Wigler et al (1979). The cells were plated in 60 mm plastic dishes at a density of 5 x 10^5 cells/plate, incubated at 37°C overnight and the medium changed before transfection. The pSV_2neo or $pSWO_4$ plasmid DNA were each added at 0.1 - 1.0 ug/ml for 16 hours at 37°C. The cells were then incubated at 37°C in regular medium for three days before switching to selective medium that contained 1 mg/ml G418 (Geneticin, Sigma Chemical Co., St. Louis, MO).

Those cells, stably transfected with either plasmid, would develop resistance to Geneticin and survive to form colonies. Three pSV_2neo-transfected V79 clones (N1-3) and six $pSWO_4$-transfected clones (MRas 1,3,5,6,7 and 20) were picked at random and utilized for the experiments performed in this study.

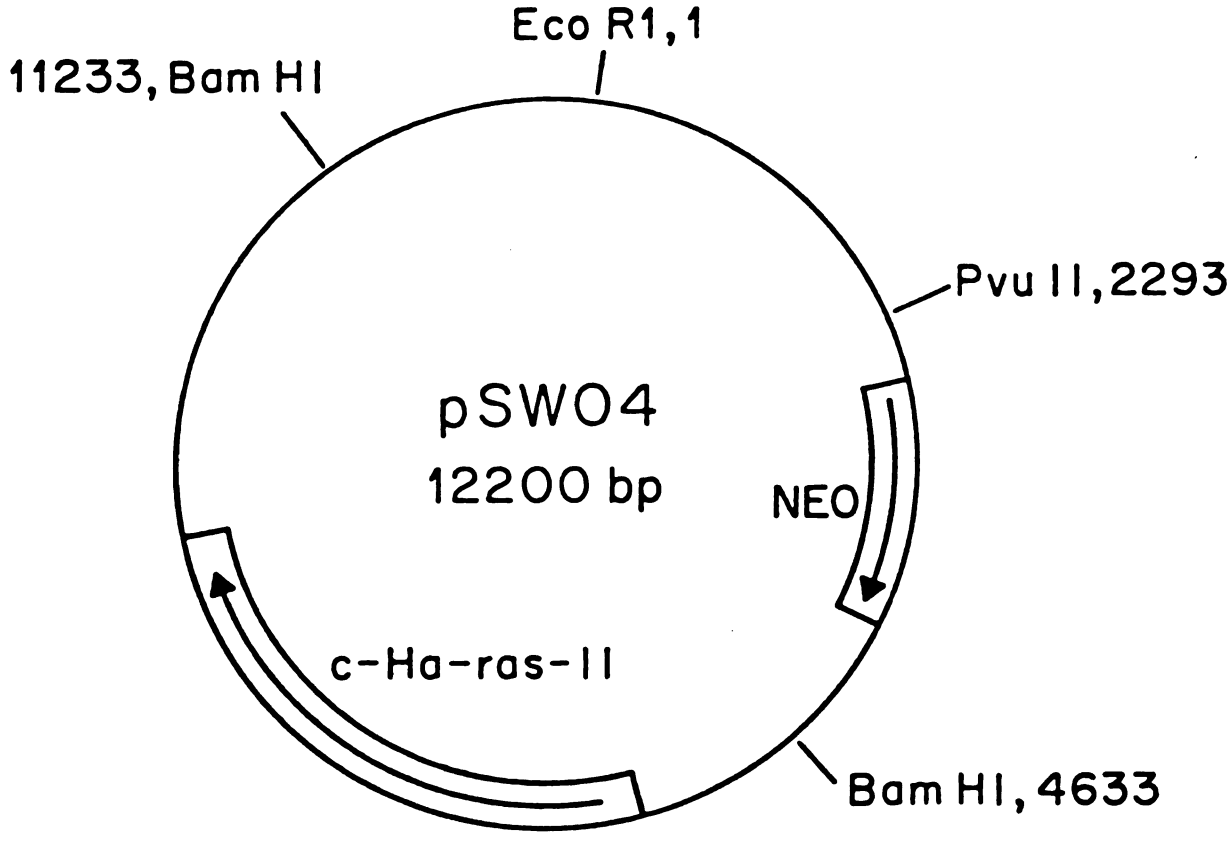


Figure 1. Diagrammatic representation of $pSWO_4$ plasmid. This recombinant plasmid (obtained from Dr. S.T. Warren, Emory University, Atlanta, GA) has a 6.6 kb BamHI fragment containing the entire human c-Ha-ras-1 oncogene from EJ/T4 carcinoma line inserted into the BamHI site of pSV_2neo. Additional PvuII sites were recognized in both the pSV_neo parental plasmid and the 6.6 kb insert upon double digestion with BamHI/PvuII restriction endonucleases (see Figure 3). The exact locations of these sites were not determined on the supplied map presented here and were irrelevant to the purpose of H-ras probe isolation. The approximate sizes of the fragments obtained are indicated in Figure 3.

c-Ha-ras-1 Probe Isolation

The $pSWO_4$ is a 12.2 Kb plasmid that contains a 6.6 Kb c-Ha-ras-1 fragment inserted at the BamHI site. It would thus be somewhat difficult to isolate the ras-containing fragment if BamHI were utilized alone. Therefore, $pSWO_4$ was digested by two restriction endonuclease enzymes, BamHI and PvuII, and electrophoresed on an agarose gel. The gel contained Hind III-digested lambda phage as a molecular size marker in addition to both pSV_2neo and $pSWO_4$, each digested or undigested with BamHI and/or PvuII (Figure 3, p. 43). The analysis indicated that the c-Ha-ras-1 gene is contained within three DNA fragments (1.5, 2.3 and 2.8 Kb). A large scale agarose gel (0.8% agarose in TBE) was prepared to isolate those fragments. A total of 100 ug $pSWO_4$ DNA was digested to completion with 500 units of each of BamHI and PvuII in buffer solution and electrophoresed. The gel was stained with 1 ug/ml ethidium bromide for 20 minutes at room temperature, the fragments were visualized and sliced out under UV light. The DNA contained in the gel slices was eluted off the agar by electroelution. The dialysis bags were electrophoresed in TBE at 70-80 volts for two hours then the DNA-containing buffer solution was filtered through siliconized glass wool and collected in plastic tubes. DNA was then extracted in succession with phenol, phenol/chloroform, butanol, and anhydrous ether. The dried pellet of DNA was dissolved in sodium acetate and absolute ethanol and kept at -20°C overnight before it was centrifuged and pelletted for 30 minutes. The DNA precipitate was finally dissolved in $T_{10}E_1$ buffer (10mM Tris, 1mM EDTA).

Extraction of Genomic DNA

Cells were grown to confluency in 150 cm^2 flasks, trypsinized and collected by centrifugation. The cells were suspended in 2 ml SDS-pronase solution at a density of $10x10^6$ - 10^8 cells/ml and incubated at 50^oC in a waterbath for three hours with frequent shaking. The DNA was extracted according to the procedure detailed in Maniatis et al (1982). Successive extractions in phenol, phenol/chloroform and chloroform were followed by ether treatment then sodium acetate and absolute ethanol to precipitate the dissolved DNA. The precipitate was kept at -20^oC overnight, resuspended in cold ethanol then ultrafuged for 15 minutes. The DNA pellet was then dissolved in $T_{10}E_1$ buffer and treated with RNAse and proteinase K for further DNA purification. The DNA was again extracted in phenol and ether than vaccumn dried and resuspended in $T_{10}E_1$ buffer.

Estimation of DNA Concentration by Spectrophotometry

A sample of the DNA solution was read in a spectrophotometer at both 260 nm and 280 nm wavelengths. An optical density (OD) of 1 equals about 50 ug DNA/ml and an OD 260/OD 280 ratio of 1.8 indicates a pure preparation of DNA.

Southern Analysis

Genomic DNA extracted from the wild type V79 cells and from six $pSW0_4$/c-H-ras transfected clones were separately digested to completion with the restriction endonuclease BamHI at 37^oC for 2-3 hours. Seven micrograms of each DNA digest was then electrophoresed in 0.8 percent agarose gel at 60 volts. The gel included a lane with the HindIII

digested lambda phage as a molecular size marker. The gel was stained with ethidium bromide and visualized with UV light. The DNA was then exposed to alkaline denaturation (0.3M NaOH and 1.5M NaCl) for one hour and neutralization in 0.5M Tris and 3M NaCl for 1.5 hours. DNA blotting on nitrocellulose filter was performed by the method of Southern (1975) and the completion of transfer was verified by UV light examination. The filter was vacuum dried and baked at 80^{o} for two hours before hybridization. The c-Ha-ras-1 probe consisted of a mixture of all three DNA fragments isolated form $pSWO_4$. These fragments were nick translated using an oligolabeling kit (Pharmacia, Inc., Piscataway, NJ). Oligolabeling was performed according to manufacturer's specifications using heat denatured probe DNA, bovine serum albumin, klenow fragment of DNA polymerase I, reagent mix with dATP, dGTP, dTTP, pd(N)6 and buffer, in addition to [α -^{32}P] dCTP (3000 Ci/mmol) which was purchased from DuPont NEN Research Products, Wilmington, DE. The oligolabeling mixture was incubated for 2 hours at room temperature, then the reactions were terminated using a stop buffer which contained dCTP, EDTA, SDS and NaCl. Hybridization was carried out for 12 hours at 42^{o}C in a water bath. The nitrocellulose filter was first washed in buffer (2x SSC and 1x Denhardts solutions) for 30 minutes at room temperature, then in 0.1x SSC and 0.1x SDS solutions for 90 minutes at 50^{o}C. The filter was then blotted dry and exposed to an x-ray film at -70^{o}C for 48 hours. The x-ray film was developed and photographed.

Detection of p21 Oncogene Product by Indirect Immunofluorescence

Wild type cells, as well as cells transfected with pSV_2neo and $pSWO_4$ and their $6TG^r$ derivatives, were examined for the expression of

the ras protein. Cells were fixed in 5% acetic acid in ethanol for 15 minutes at -20°C, then air dried and incubated at 37°C for 60 minutes with 20 ug/ml V-H-ras (Ab-1) monoclonal antibody, clone Y13-259 (Furth 1982), purchased from Oncogene Science, Inc., Mineola, NY). Cells were washed in PBS and reincubated at 37°C with goat anti-rat IgG FITC conjugate (Sigma Chemical Co., St. Louis, MO) for 30-45 minutes in a dark moist chamber. The cells were then washed in PBS and examined under epifluorescence microscopy.

Isolation of $6TG^r$ Cells

The V79 $6TG^s$ wild type cells and those transfected with H-ras were exposed to a total of 1000 rads of x-rays, then reincubated at 37°C in humidified air and 5 percent CO_2 in several changes of fresh medium for about one week. Subsequently, 6TG (10 ug/ml) was added to the medium to select for $6TG^r$ mutants. The surviving mutants were seeded in 9cm plastic dishes at a density of 100 cells/dish. Individual colonies were then isolated at random and propagated. Mutant ($6TG^r$) cells were thus obtained from wild type V79 cells and from different H-ras transfected clones. Three $6TG^r$ subclones were isolated from each of the six H-ras transfected clones and used in the co-culture combinations described below.

The Metabolic Cooperation Assay

Plating efficiency

Cells from each of the $6TG^r$ clones, which included V79R (the $6TG^r$ cells derived from the wild type V79 cells) and all $6TG^r$ subclones derived from the six H-ras transfected clones, were tested for their

plating efficiency. This was determined by seeding 100 cells of each type in 60mm plastic dishes (5 plates/cell type) and culturing in similar medium , 6TG concentration, and incubation conditions as described before. At the end of ten days the colonies were fixed and stained with Giemsa and scored. An average count was calculated for each $6TG^r$ clone and considered as a base line, 100 percent relative plating efficiency.

Determination of Corrected Percentage of the $6TG^r$ Clones Recovered in the Co-culture Experiments.

For each co-culture combination, ten plates were seeded each with 100 $6TG^r$ cells as described in the next section. The surviving colonies were counted in all ten plates and an average count was calculated and then corrected for the corresponding plating efficiency of the $6TG^r$ cell type utilized.

Co-culture Combinations

For these combinations, 4 x 10^5 $HPRT^+$ (i.e. $6TG^s$) cells were co-cultured with 100 $6TG^r$ cells in the presence of 10 ug/ml 6-thioguanine. Under these conditions, the $6TG^r$ cells could only survive if they were uncoupled from the $6TG^s$ ones.

Preliminary experiments were performed on Chinese hamster cells already transfected with c-Ha-ras-1 (provided by Dr. S.T. Warren). These clones have been tested for c-Ha-ras-1 integration in the genomic DNA (Warren, unpublished results). Two of these clones (x2 and x5) were utilized in the metabolic cooperation assay. Mutant (i.e. $6TG^r$) subclones were derived from each one by x-irradiation as described

before. Three $6TG^r$ subclones were selected at random from each clone. These subclones, designated x2R6, X2R9, X2R10 and X5R3, X5R7, X5R16, were derived from X2 and X5 respectively. Plating efficiency was determined for each of the $6TG^r$ cell type utilized including the V79R derived from the wild type. The co-culture combinations (six plates each) were set as listed in Table 1.

Table 1. Co-cultured combinations in the metabolic cooperation assay of the x2 and x5 c-Ha-ras-1 transfected V79 cells.

$6TG^s$	Cells/plate	$6TG^r$	Cells/plate
Control			
V79	$4x10^5$*	V79R	100*
Experiments			
X2	$4x10^5$	V79R	100
X2	$4x10^5$	X2R6	100
X2	$4x10^5$	X2R9	100
X2	$4x10^5$	X2R10	100
V79	$4x10^5$	X2R6	100
V79	$4x10^5$	X2R9	100
V79	$4x10^5$	X2R10	100
X5	$4x10^5$	V79R	100
X5	$4x10^5$	X5R3	100
X5	$4x10^5$	X5R7	100
X5	$4x10^5$	X5R16	100
V79	$4x10^5$	X5R3	100
V79	$4x10^5$	X5R7	100
V79	$4x10^5$	X5R16	100

*Cells were counted in a hemocytometer and diluted to approximately $4x10^5$/ml $6TG^s$ and 100/ml $6TG^r$ cells. Each plate received one ml of the two different types of cells.

A more comprehensive study was planned and wild type V79 cells were newly transfected with either pSV_2neo or $pSWO_4$ plasmids by calcium phosphate precipitation as described before. Transfectants were selected in G418 and three clones of pSV_2neo and six of $pSWO_4$ transfected cells were picked at random and propagated separately. From each of the $pSWO_4$, c-Ha-ras-1 containing clones, three $6TG^r$ mutants were induced by x-irradiation as described. For control, a $6TG^r$ mutant (V79R), derived from the wild type V79 was co-cultured with the parental $6TG^s$ cells, as well as with six clones derived from the same parental pool to test for homogeneity of gap junctional communication competence. The co-cultures, controls and ras transfected cells are listed in Tables 2 and 3. These experiments were repeated twice under similar conditions.

Table 2. Control combinations in the metabolic cooperation assay for the x2 and x5 c-Ha-ras-1 transfected V79 cells.

$6TG^s$		Cells/plate	$6TG^r$	Cells/plate
<u>Wild type V79</u>				
heterogenous V79		$4x10^5$*	V79R	100*
Clone 1	(C1)	$4x10^5$	V79R	100
Clone 2	(C2)	$4x10^5$	V79R	100
Clone 3	(C3)	$4x10^5$	V79R	100
Clone 4	(C4)	$4x10^5$	V79R	100
Clone 5	(C5)	$4x10^5$	V79R	100
Clone 6	(C6)	$4x10^5$	V79R	100
<u>pSV_2neo-transfected cells</u>				
Clone 1	(N1)	$4x10^5$	V79R	100
Clone 2	(N2)	$4x10^5$	V79R	100
Clone 3	(N3)	$4x10^5$	V79R	100

*Cells were counted with a hemocytometer and diluted to approximately $4x10^5$/ml $6TG^s$ and 100/ml $6TG^r$. Each plate received one ml of the two different types of cells.

Table 3. The metabolic cooperation assay: Co-cultured combinations of $6TG^S$ of H-ras transfected cells with $6TG^r$ of untransfected (V79R) or H-ras transfected (MRasR) cells.

$6TG^S$	Cells/plate	$6TG^r$	Cells/plate
MRas1	$4x10^5$*	V79R	100*
MRas1	$4x10^5$	MRas1 R1**	100
MRas1	$4x10^5$	MRas1 R2	100
MRas1	$4x10^5$	MRas1 R3	100
MRas3	$4x10^5$	V79R	100
MRas3	$4x10^5$	MRas3 R1	100
MRas3	$4x10^5$	MRas3 R2	100
MRas3	$4x10^5$	MRas3 R3	100
MRas5	$4x10^5$	V79R	100
MRas5	$4x10^5$	MRas5 R1	100
$MRas^5$	$4x10^5$	MRas5 R2	100
MRas5	$4x10^5$	MRas5 R3	100
MRas6	$4x10^5$	V79R	100
MRas6	$4x10^5$	MRas6 R1	100
$MRas^6$	$4x10^5$	MRas6 R2	100
MRas6	4x105	MRas6 R3	100
MRas7	$4x10^5$	V79R	100
MRas7	$4x10^5$	MRas7 R1	100
MRas7	$4x10^5$	MRas7 R2	100
MRas7	$4x10^5$	MRas7 R3	100
MRas20	$4x10^5$	V79R	100
MRas20	$4x10^5$	MRas20 R1	100
MRas20	$4x10^5$	MRas20 R2	100
MRas20	$4x10^5$	MRas20 R3	100

*Cells were counted in a hemocytometer and diluted to approximately $4x10^5$/ml $6TG^S$ and 100/ml of $6TG^r$.

** Three $6TG^r$ subclones were derived from each of the ras-transfected $HPRT^+$ clones.

Scrape-Loading/Dye Transfer Assay

This new assay was developed during the course of this work. Its working hypothesis was derived from an observation made by McNeil et al (reference 27 p. 68) when they successfully introduced macromolecules intracellularly by scraping cells grown in vitro. A complete description and applications are enclosed on pages 61-79 (El-Fouly et al 1987, Suter et al 1987). This assay involves the introduction of a membrane impermeable fluorescent tracer (i.e. Lucifer yellow, MW 457.2) intracellularly by scrape-loading the cells in a monolayer culture. The dye's relatively small molecular weight allows its free passage across patent gap junctions into contiguous cells. In addition to Lucifer yellow, a second high molecular weight marker dye (e.g. rhodamine dextran, MW 10,000) could be concurrently loaded to label, and thus identify, the primary-loaded cells. The rhodamine dextran, which emits red fluorescence, is a membrane, as well as gap junction, impermeable dye.

The cells are plated in 35 mm plastic dishes at a density that allows a confluent monolayer to be formed within 12-18 hours. The incubation conditions are similar to those described above and the medium should be appropriate to the cell type. The cells are rinsed with PBS before the addition of two milliliters of 0.05% Lucifer yellow and rhodamine dextran (purchased from Molecular Probes, Inc. Eugene, Oregon) dissolved in PBS. The cells are then scraped in parallel lines in the presence of the dye mixture with a sharp edge or a wooden probe. The dye solution is left on the cells for 1-2 minutes, then discarded and the plates rinsed with PBS to remove detached cells, debris, and background fluorescence. Fresh medium is added and the cells are examined under an epifluorescence microscope. Control plates, including wild

type V79 cells and cells transfected with $pSV0_2neo$ alone were tested for the extent of their communication ability. In addition, all the $pSW0_4$/H-ras transfected cells and their $6TG^r$ derivatives were examined under similar conditions for dye transfer. The extent of communication was measured by counting the number of the secondary dye-recipient cells in a fixed surface area under epifluorescence microscopy.

RESULTS

Transfection of V79 Cells

The V79 cells were transfected with ras plasmid DNA by calcium phosphate precipitation and followed by selection in G418. Resistant colonies were formed in about 10-14 days at a frequency of 10-15 colonies per ug plasmid DNA per 5 x 10^5 cells. Three pSV_2neo and six $pSWO_4$ transfected colonies were selected at random and propagated for further studies. All the clones derived from the pSV_2neo transfected cells had morphology comparable to that of the wild type V79 cells and grew in homogenous monolayers (Figure 2a,b). Those clones transfected with $pSWO_4$ showed cells with morphological and growth variations (Figure 2c). These included spindle-like shape, contact insensitivity and poor attachment to the substrate. Both pSV_2neo and $pSWO_4$ transfected cells were maintained under selective pressure in media containing 0.5-1mg/ml G418 throughout the duration of the experiments.

c-Ha-ras-1 Probe Isolation

Digestion of $pSWO_4$ with both BamHI and PvuII restriction endonucleases resulted in multiple fragments following electrophoresis (Figure 3). When compared to the fragments obtained by digesting the pSV_2neo plasmid with the same enzymes, three unique fragments were identified (1.5, 2.3 and 2.8 Kb) that contained the entire c-Ha-ras-1 gene. These fragments were subsequently isolated from a large scale agarose gel purified and utilized as probe in Southern blotting.

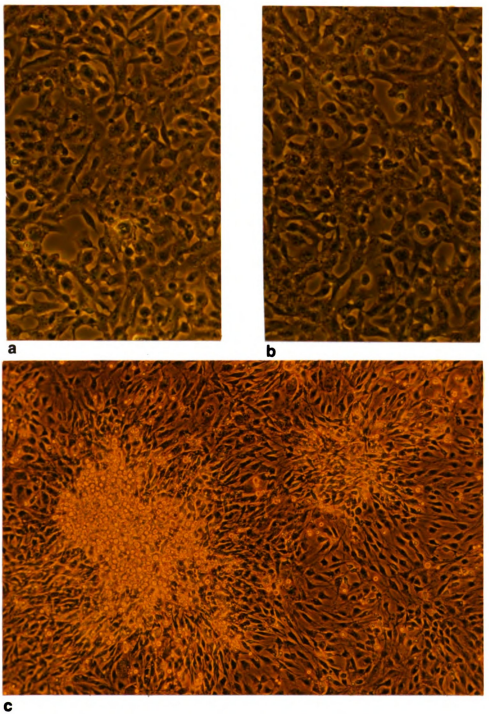


Figure 2. Morphological features of the V79 cells before and after transfection with pSV_2neo or $pSW0_4$ plasmids. a. Wild type cells. b. Cells transfected with pSV_2neo. c. Cells transfected with $pSW0_4$ containing c-Ha-ras-1 oncogene.

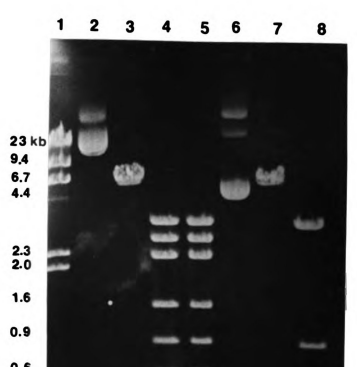


Figure 3. Agarose gel electrophoresis and ethidium bromide staining of pSV_2neo and $pSWO_4$ plasmid digests. Electrophoresis of BamHI/PvuII double digest of a pSV_2neo and $pSWO_4$ resulted in a total of five and eight fragments respectively. The smallest of these fragments, (a 0.24 kb common to both plasmids) was not retained in the above gel. These fragments revealed the presence of four PvuII sites in the pSV_2neo region and two in the 6.6 kb insert. The exact locations of these sites were not determined in the provided $pSWO_4$ map in Figure 1 and were not critical for the purpose of isolating the H-ras probe. The double digest of $pSWO_4$ with BamHI/PvuII endonucleases showed, upon electrophoresis, three additional fragments (2.8 kb, 2.3 kb and 1.5 kb) when compared to the similarly digested parental pSV_2neo plasmid. These extra fragments, combined, contain the 6.6 kb c-Ha-ras-1 insert. These H-ras-containing fragments were excised from the gel, their DNA extracted, combined and utilized as probe in the detection of the c-Ha-ras-1 as described in Materials and Methods. Lane 1, lambda phage Hind III digest used as molecular weight marker. Lane 2, $pSWO_4$ undigested. Lane 3, $pSWO_4$ BamHI digested. Lane 4 and 5, are duplicate of $pSWO_4$ double digested with BamHI/PvuII. Lane 6, pSV_2neo undigested. Lane 7, pSV_2neo digested with BamHI. Lane 8, pSV_2neo double digested with BamHI/PvuII.

DNA Analysis by Southern Transfer

The presence of c-Ha-ras-1 was confirmed by Southern analysis, using as probe the three DNA fragments that contained the entire gene. The ras-specific probe intensely hybridized to a common 6.6 Kb band in all ras-transfected clones tested (Figure 4, lanes 2-7). In addition, hybridization also occurred in adjacent bands indicative of integration of more than one copies of the ras oncogene. The probe also detected a faint band of similar size in the wild type V79 cells. This band may represent the endogenous H-ras homolog in the hamster cells. (Figure 4, lane 1). The molecular size of the bands was determined by comparison to a HindIII digested lambda marker electrophoresed on the same gel prior to blotting.

Detection of c-Ha-ras-1 Expression by Indirect Immunofluorescence

All six clones transfected with $pSWO_4$, as well as the $6TG^r$ mutant, subclones derived from each one by x-irradiation, showed strong fluorescence after antibody treatment as described in Methods. The fluorescence was most prominent in the cell membranes and in the cytoplasms. The nuclei appeared less fluorescent in all cells examined (Figure 5). The wild type cells and those transfected with pSV_2neo alone showed no fluorescence or a faint trace in the cytoplasm of few of the cells examined (Figure 6).

The Metabolic Cooperation Assay

The results obtained from the preliminary experiments utilizing the $pSWO_4$-transfected x2 and x5 clones are presented in Figure 7. The data indicate a significant increase in the recovery of $6TG^r$ cells over

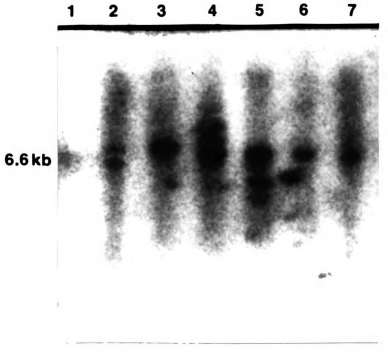
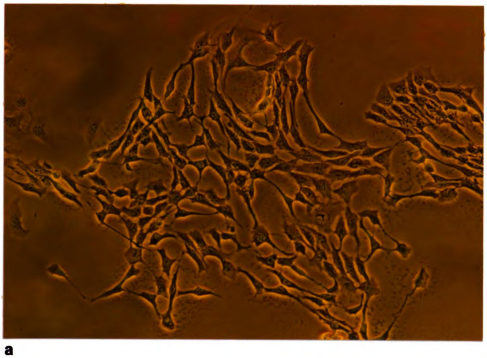
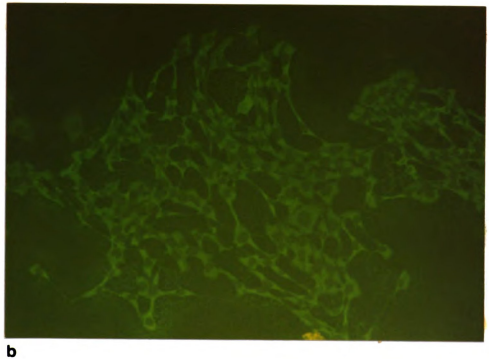


Figure 4. Southern blot analysis. As described in Materials and Methods, genomic DNA extracted from wild type V79 cells and cells transfected with $pSW0_4$ were digested to completion with BamHI. Seven micrograms of digested DNA were electrophoresed in each lane of an agarose gel, blotted on nitrocellulose filter and hybridized to a ^{32}P-labelled c-Ha-ras-1 probe with a specific activity of $4.5x10^8$ cpm/ug. Hybridization was carried overnight at 42^oC. The nitrocellulose filter was soaked in the first wash solution of 1X SSC and 1X Denhardt's in distilled water for one hour at room temperature. A second wash was done in 0.1X SSC and 0.1% SDS in distilled water at 50^oC for 1.5 hours. The nitrocellulose filter was then dried between sheets of 3M paper at room temperature then autoradiographed by exposure to an x-ray film at -70^oC for 48 hours. The film was developed and photographed. Lane 1, wild type V79 cells; lanes 2-7, genomic DNA extracted from MRas 1,3,5, 6,7 and 20 respectively.



a



b

Figure 5. Detection of the c-Ha-ras-1 product (p21) by indirect immunofluorescence using monoclonal antibody in H-ras transfected cells. All six clones of c-Ha-ras-1 transfected cells were tested and showed the same pattern of fluorescence depicted in this figure of the MRas7 clone. a. Phase contrast photomicrograph. b. Epifluorescence of the same field as in a.

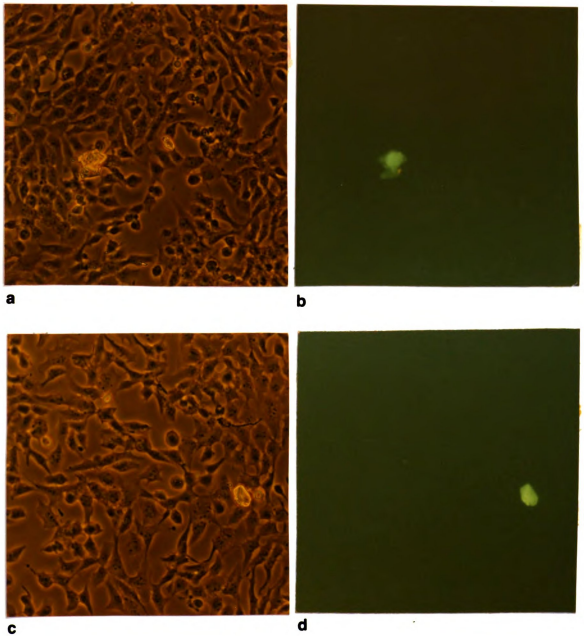


Figure 6. Detection of p21 product by indirect immunofluorescence using monoclonal antibody in wild type cells and cells transfected with the control plasmid pSV_2neo. a. Wild type V79 cells under phase microscopy. b. Same field as in a, under epifluorescence. c. V79 cells transfected with pSV_2neo. d. Same field as in c, under epifluorescence.

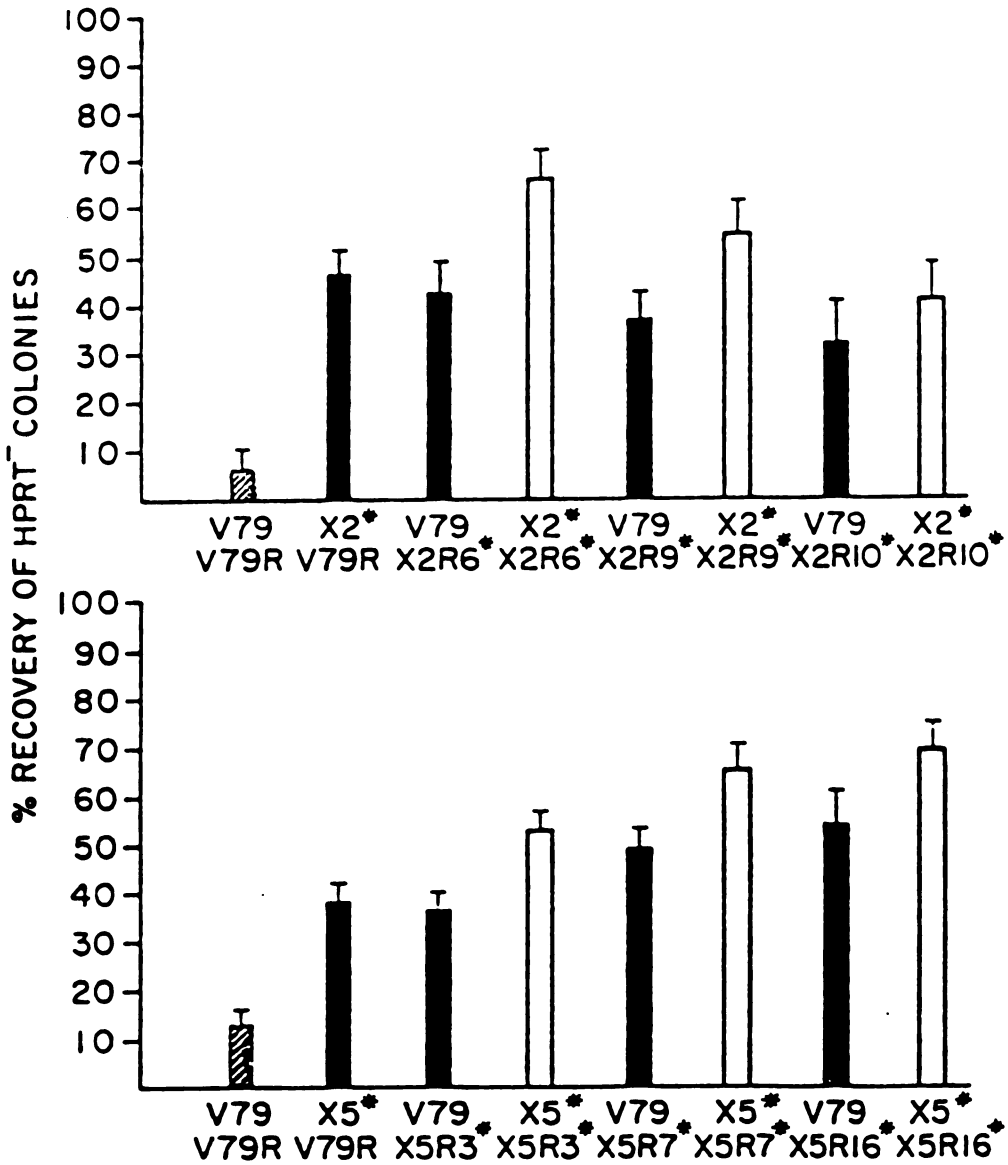


Figure 7. Co-cultured combinations in the metabolic cooperation assay of the x2 and x5 c-Ha-ras-1 transfected V79 cells. All cell clones utilized were transfected with H-ras with the exception of the $6TG^S$ wild type V79 cells and the $6TG^r$, V79R. The bars indicate the percent of $6TG^r$ cell recovery with (*), or without (V79R), ras transfection in co-cultured combinations. Solid and open bars represent co-cultures in which only one or both cell types transfected with H-ras respectively. The hatched bars represent control combinations. The x2 and x5 clones are $6TG^S$ ras-transfected V79 cells from which the $6TG^r$ subclones, X2r6,9,10 and X5R3,7 and 16 were derived respectively.

the control value. This was true when either or both $6TG^S$ and/or $6TG^r$ cells were transfected with c-Ha-ras-1 plasmid. Statistical analysis using t-test indicates significant difference in the recovery of $6TG^r$ cells transfected with c-Ha-ras-1 as compared to the control ($p < 0.0005$, Table 4).

The two comprehensive metabolic cooperation studies included control series that consisted of pooled wild type and six randomly selected wild type clones, in addition to cells transfected with pSV_2neo alone and 24 different co-cultured combinations of c-Ha-ras-1 transfected cells. The results are presented in Figures 8 and 9 and Table 5. The data and the statistical analyses using Student t-statistics indicate no significant difference in the recovery of the V79 ($6TG^r$) cells when co-cultured with pSV_2neo-transfected cells as compared to control cells without transfection. In all cells transfected with c-Ha-ras-1 the recovery of $6TG^r$ was significantly different from all the control series ($p < 0.00001$).

Table 4. Statistical analysis of the metabolic cooperation assay performed with the x2 and x5 c-Ha-ras-1 transfected clones.

Co-cultured cells	% recovery of $6TG^r$ u(± SE)	t(observed)
Control		
V79/V79R[1]	[$\bar{x}_1$] 7.07 (0.66)	—
V79/V79R[2]	[$\bar{x}_2$]13.70 (1.00)	—
Experiments		
X2/V79R	46.88 (1.44)	27.65*
V79/X2R6	43.23 (2.36)	15.32*
X2/X2R6	66.75 (1.93)	30.92*
V79/X2R9	38.09 (2.50)	12.41*
X2/X2R9	55.38 (1.98)	24.40*
V79/X2R10	34.39 (1.73)	15.79*
X2/X2R10	41.46 (2.63)	13.08*
X5/V79R	39.03 (2.15)	11.78*
V79/X5R3	36.72 (2.74)	8.40*
X5/X5R3	54.04 (1.93)	20.90*
V79/X5R7	50.00 (1.94)	18.71*
X5/X5R7	63.02 (2.44)	20.21*
V79/X5R16	55.69 (1.48)	28.37*
X5/X5R16	71.05 (1.77)	32.40*

1. Control combination obtained for X2 clone experiments.
2. Control combination obtained for X5 clone experiments.
u: mean of sample distribution (% recovery of $6TG^r$).
$\bar{x}$: Mean of control distribution (% recovery of $6TG^r$).

$$t(\text{observed}) = \frac{u - \bar{x}_1 \quad (\text{or } \bar{x}_2)}{\text{SE of sample distribution}}$$

*: t(critical) = 7.98 at df=5 and p=0.0005

Table 5. Statistical analysis (t-test) of experiments I and II of Metabolic Cooperation

Co-cultured cells	Experiment I		Experiment II	
	u (± SE)	t(observed)	u (± SE)	t(observed)
Control				
V79/V79R (pooled)	[$\bar{x}$]14.11(1.16)	———	[$\bar{x}$]13.09(0.63)	———
/N_1(V79R+pSV$_2$neo)	14.57(0.80)	0.58	15.68(1.12)	2.31
/N_2(V79R+pSV$_2$neo)	16.18(1.14)	1.82	17.65(1.62)	2.81
/N_3(V79R+pSV$_2$neo)	16.44(0.98)	2.38	14.62(0.88)	1.74
Ras-transfected				
MRas1/V79R	59.63(1.63)	27.93*	57.18(1.34)	32.90*
/MRas1R1	87.08(1.28)	57.01*	70.91(0.94)	61.51*
/MRas1R2	77.76(2.28)	27.92*	75.34(1.66)	37.50*
/MRas1R3	76.05(1.71)	36.22*	72.00(1.28)	46.02*
MRas3/V79R	80.08(1.44)	45.81*	79.84(1.99)	33.54*
/MRas1R1	84.14(1.50)	46.69*	83.40(1.43)	49.17*
/MRas1R2	90.32(1.70)	44.83*	80.17(1.35)	49.69*
/MRas1R3	87.06(1.67)	43.68*	82.94(1.82)	38.38*
MRas5/V79R	61.76(1.52)	31.35*	56.92(1.49)	29.42*
/MRas5R1	76.66(1.19)	52.56*	72.07(2.13)	27.69*
/MRas5R2	72.57(2.42)	24.16*	73.09(1.11)	54.05*
/MRas5R3	77.42(1.55)	40.85*	67.61(1.69)	32.26*
MRas6/V79R	81.63(1.58)	42.73*	77.60(1.67)	38.63*
/MRas6R1	82.82(1.60)	42.94*	91.70(1.79)	43.92*
/MRas6R2	94.62(2.15)	37.45*	85.48(1.30)	55.68*
/MRas6R3	91.21(1.57)	49.15*	89.58(1.64)	46.64*

Table 5 (cont'd.)

Co-cultured cells	Experiment I		Experiment II	
	u (± SE)	t(observed)	u (± SE)	t(observed)
MRas7/V79R	86.63(1.58)	45.90*	86.82(1.75)	42.13*
/MRas7R1	90.96(1.44)	53.37*	91.99(1.70)	46.41*
/MRas7R2	84.46(1.62)	43.43*	95.94(1.50)	55.23*
/MRas7R3	91.42(1.42)	54.44*	92.08(1.72)	45.92*
MRas20/V79R	83.16(1.47)	46.97*	75.63(1.30)	48.11*
/MRas20R1	81.51(1.65)	40.85*	80.21(1.39)	48.29*
/MRas20R2	84.19(1.39)	50.42*	93.83(1.84)	43.88*
/MRas20R3	84.52(2.15)	32.75*	83.04(1.47)	47.59*

u: mean of sample distribution (% recovery of $6TG^r$ in a total of 10 plates/co-culture combination)

$\bar{x}$: mean of control distribution (% recovery of $6TG^r$ in a total of 10 plates/co-culture combination)

$$t(\text{observed}) = \frac{u - \bar{x}}{SE \text{ (of sample distribution)}}$$

*t(critical), df=9 at p:0.01 = 2.82, p:0.005 = 3.25, p:0.00001 = 8.10

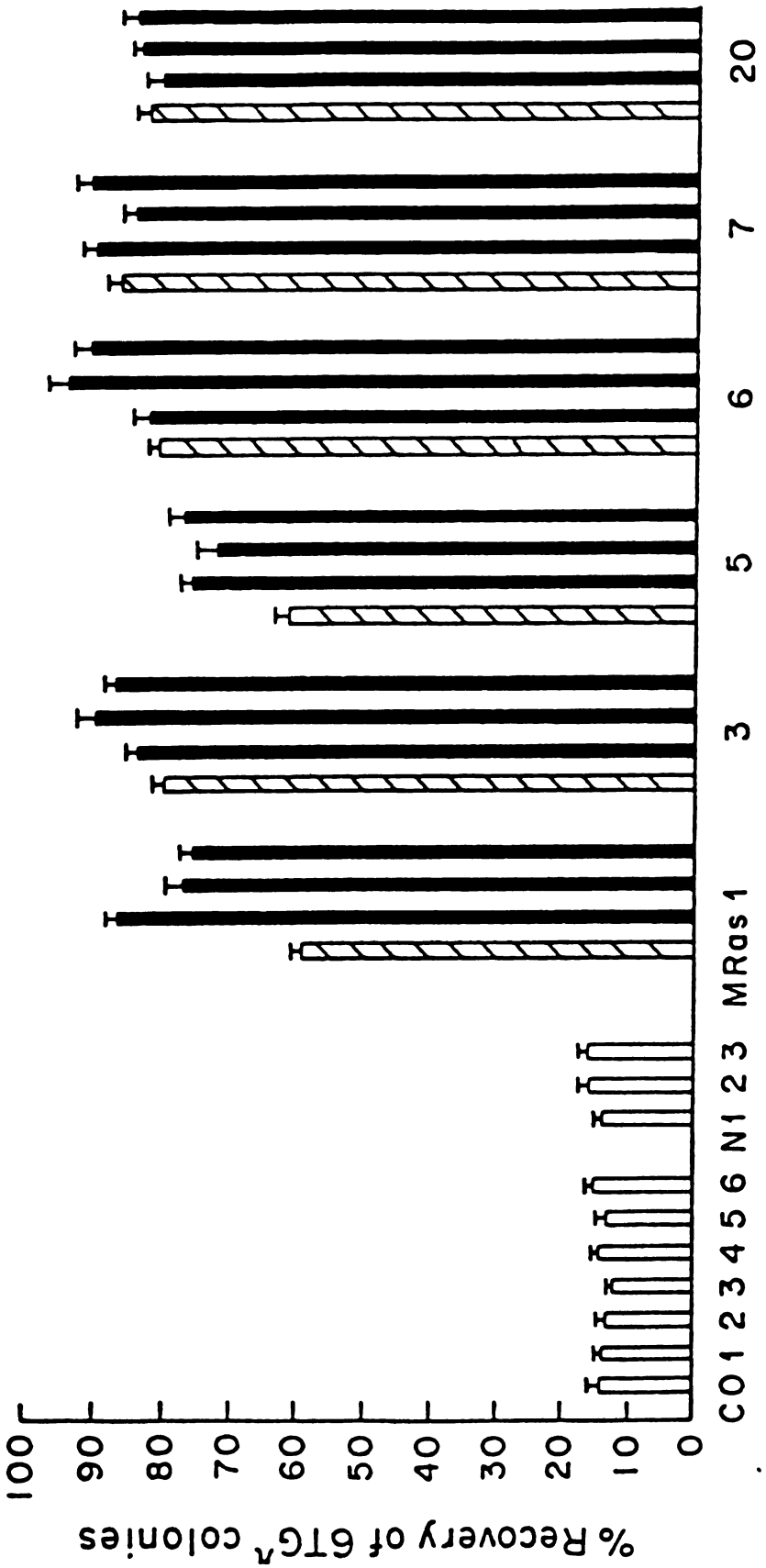


Figure 8. Co-cultured combinations of $6TG^s$ and $6TG^r$ cells with and without c-Ha-ras-1 expression in the metabolic cooperation assay (experiment I). All bars represent the percent recovery of HPRT- cells in the presence of 6TG. The control series (open bars) included six randomly selected clones (C1-6) from the wild type V79 pool (CO), and three pSV2neo transfected cells (N1-3). Six ras-transfected $6TG^s$ clones, MRas1,3,5,6,7,20 co-cultured with either non-transfected (hatched bars) or with each of three $6TG^r$ (ras-transfected) subclones derived from the same parental ras-transfected cells (solid bars).

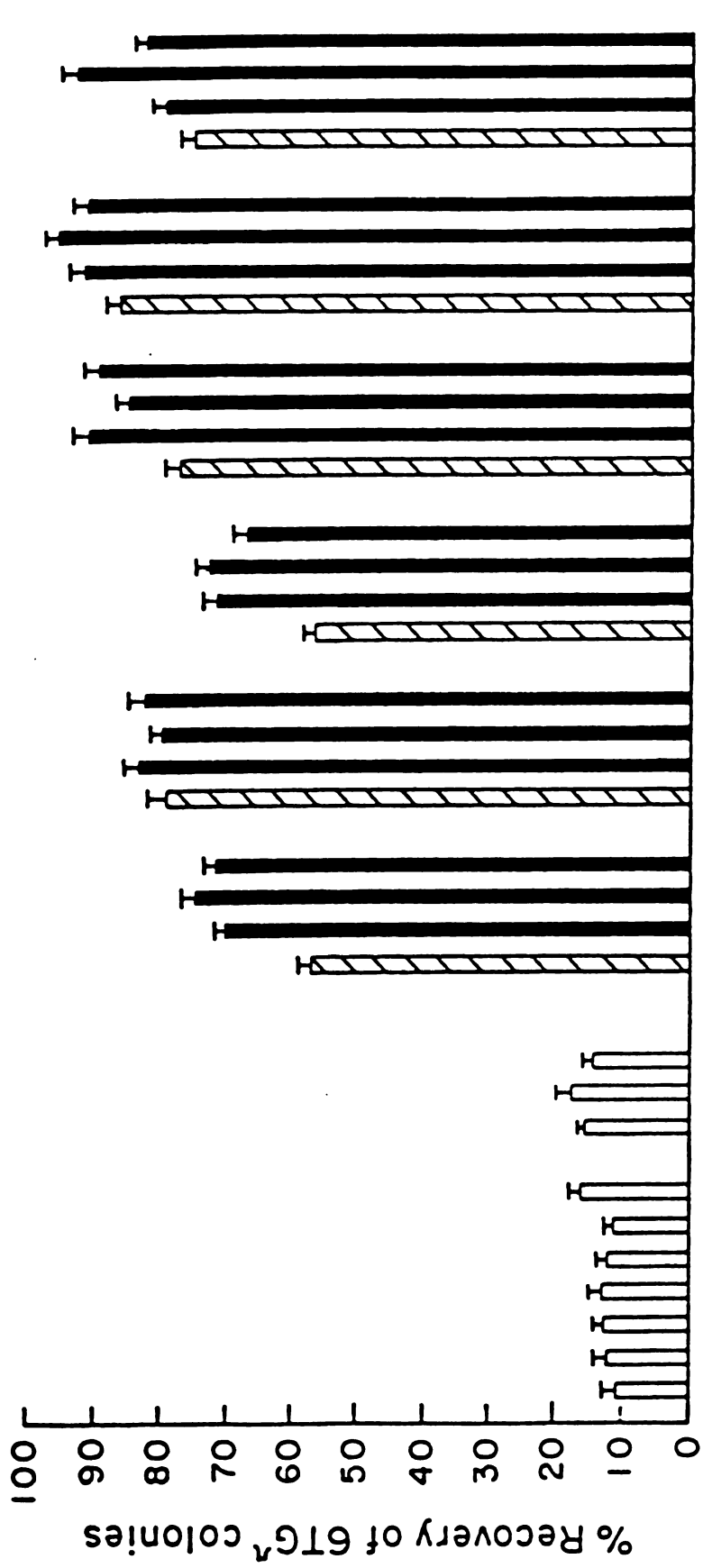


Figure 9. Co-cultured combinations of $6TG^S$ and $6TG^r$ cells with and without c-Ha-ras-1 expression in the metabolic cooperation assay (experiment II). All bars represent the percent recovery of $HPRT^-$ cells in the presence of 6TG. The control series (open bars) included six randomly selected clones (C1-6) from the wild type V79 pool (CO), and three pSV_2neo transfected cells (N1-3). Six ras-transfected $6TG^S$ clones, MRas1,3,5,6,7,20 co-cultured with either non-transfected (hatched bars) or with each of three $6TG^r$ (ras-transfected) subclones derived from the same parental ras-transfected cells (solid bars).

Scrape-Loading/Dye Transfer Assay

This newly developed assay, derived from a method to introduce macro-molecules into cells in culture (reference 27 p. 68), was tested with cells of different types and from different organisms with and without exposure to chemicals known to block intercellular communication (El-Fouly et al 1987). The results were in agreement with previous studies. The loading of membrane impermeable fluorescent dyes intracellularly was achieved by scraping cells, grown in monolayer cultures as described in Materials and Methods. In primary cell cultures, e.g. human fibroblasts and keratinocytes and in established cell lines known for their gap junctional competence, the low MW Lucifer yellow (LY) dye diffused within seconds into contiguous cells. The extent of dye transfer varied from one cell type to another. Figure 10 represents the degree of dye transfer in human primary fibroblasts and Figure 11 shows primary human keratinocytes loaded with both LY and rhodamine dextran. The rhodamine dextran molecules (MW 10,000) being larger than permissible size limit for crossing gap junctions remains in the primary loaded cells, thus labeling those initially scrape loaded. The LY, on the other hand, was able to diffuse freely presumably across the membrane gap junction thus permeating the contiguous cells. A clear LY concentration gradient was noted being higher at the edge of the scraped cells and decreasing in intensity further away from the line of scraping. The LY diffusion into contacting cells continued over time and was diluted out and became almost undetectable in about one hour. In cells treated with chemicals known to block gap junctional intercellular communication the dye transfer was inhibited. A study on the effects of dose-response and temporal course of cell exposure to

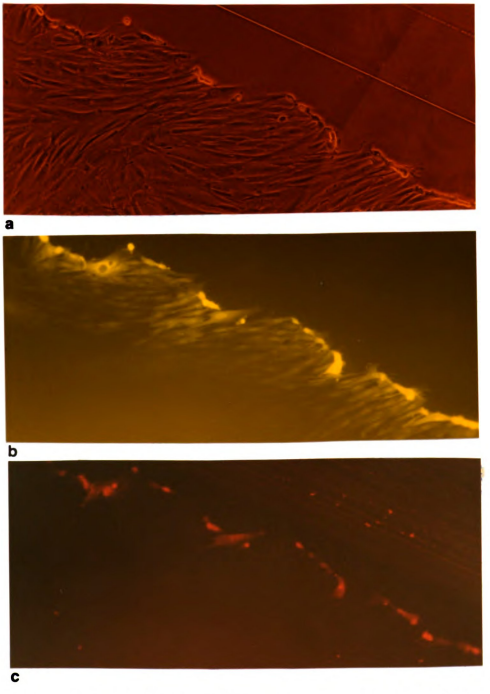


Figure 10. Gap junctional mediated dye transfer in primary human fibroblasts by the Scrape-Loading/Dye Transfer assay. The extent of junctional competence is determined by the degree of Lucifer yellow dye transfer among contiguous cells. a. Phase-contrast micrograph of human fibroblasts in culture. b. Lucifer yellow transfer to contiguous cells. c. Rhodamine dextran marker dye labelling the primary loaded cells. a, b and c are photographs of the same field using different light filters.

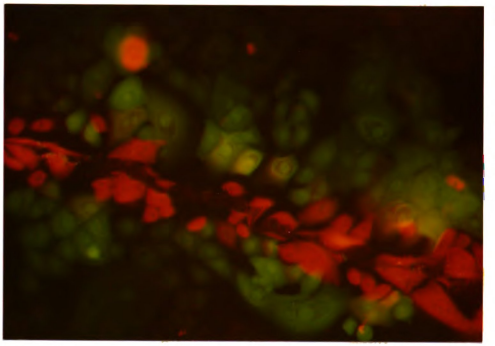


Figure 11. Gap junctional mediated dye transfer in primary human keratinocytes as detected by the Scrape-Loading/Dye Transfer assay. Primary loaded cells are labelled with the high molecular weight rhodamine dextran dye (red fluorescence). Lucifer yellow (MW 457.2) permeated coupled cells further away from the scrape line.

certain gap junction-modulating chemicals was undertaken and the results indicated the sensitivity of the assay in detecting such changes (Suter et al 1987). In the experiments done here, wild type V79 cells including the six randomly selected subclones of wild type cells and those cells transfected with pSV_2neo alone showed efficient dye transfer (Figure 12). The cells transfected with c-Ha-ras-1 (i.e., the x2 and x5) and their $6TG^r$ derivatives, in addition to the six $pSWO_4$-transfected clones, i.e. MRas1, 3, 5, 6, 7, and 20 and all their $6TG^r$ subclones showed complete inhibition of the dye transfer following scrape loading (Figure 13).

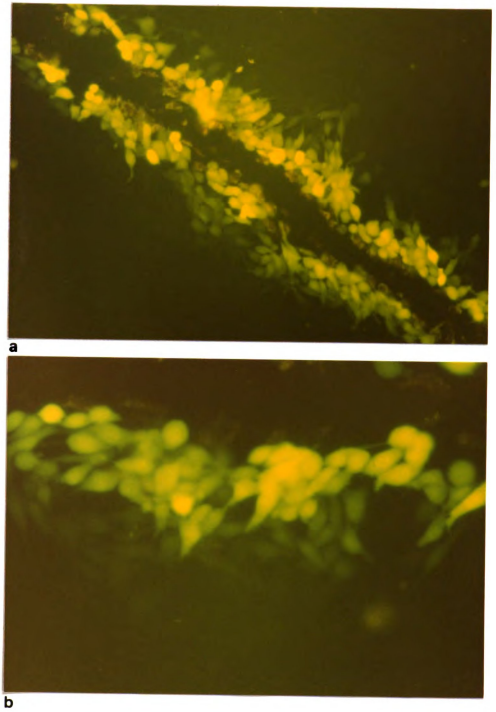


Figure 12. Gap junction-mediated dye transfer in V79 cell detected by Scrape-Loading/Dye Transfer. a. Wild type V79 cells. b. Cells transfected with pSV_2neo control plasmid.

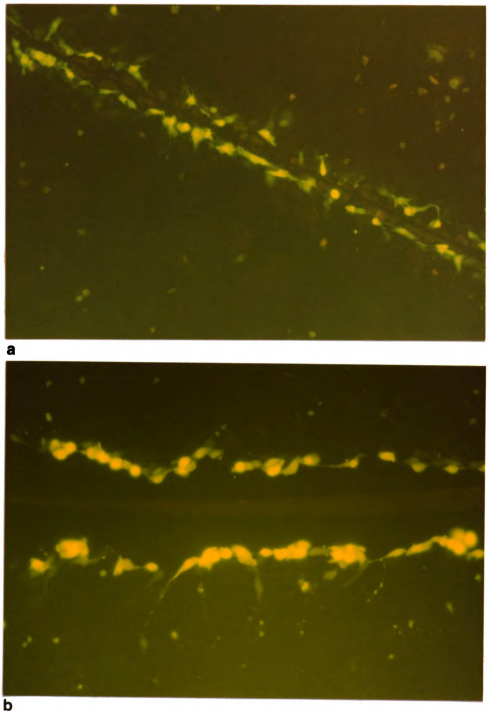


Figure 13. Inhibited gap junction-mediated dye transfer in cells transfected with the c-Ha-ras-1 oncogene. All six clones (i.e. MRas1, 3, 5, 6, 7 and 20) showed no dye transfer from the primary loaded cells to the contiguous ones. MRas1 and MRas5 are represented in a and b respectively.

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Scrape-Loading and Dye Transfer

A Rapid and Simple Technique to Study Gap Junctional Intercellular Communication

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Gap junction-mediated intercellular communication has been recognized in cells from different tissues of various organisms and has been implicated in a variety of cellular functions and dysfunctions. Here we describe a new, direct and rapid technique with which to study this cellular phenomenon. It employs scrape-loading to introduce a low molecular weight (MW) fluorescent dye, Lucifer yellow CH (MW 457.2) into cells in culture and allows the monitoring of its transfer into contiguous cells. In communication-competent cells the dye transmission occurred within minutes after loading. The involvement of membrane junctions in Lucifer yellow transfer was verified by the concurrent loading of a high MW marker dye conjugate, rhodamine dextran (MW 10000). Once introduced intracellularly the rhodamine dextran is unable to cross the relatively narrow membrane junctions. Chemicals of variable potency known to block junctional communication were tested in Chinese hamster V79 cells and other mammalian cells. The results showed effective blockage of the dye transfer at non-cytotoxic doses. This new technique can be applied to a wide variety of mammalian (including human) cells. In addition, it has the potential to be utilized as a rapid screening assay to detect chemicals that can modulate intercellular communication and to study their mechanism of action.

Gap junction-mediated intercellular communication has been considered as an important determinant for normal cell growth and differentiation [1–3]. Mammalian gap junctions permit the exchange of nutrients, ionic signals and regulatory molecules of approx. 1500 D among contacting, communication-competent cells [4]. Modulation of this phenomenon by either natural physiological states or by xenobiotics has been demonstrated in many studies [5–9]. Inhibition of this form of intercellular communication by various chemicals has been postulated to be a factor in the tumor promotion phase of carcinogenesis [10–12], teratogenesis [13–16], neurotoxicity [17], reproductive dysfunction and other chemically-induced disease states [8].

Measurement of gap-junctional communication has been achieved by using electrocoupling [18], dye transfer following microinjection [19–21], radioactive metabolite transfer [22], metabolic cooperation of cells with enzyme deficiencies in certain metabolic pathways [23–25] and FRAP analysis (fluorescence recovery after photobleaching) [26]. While each of these techniques has certain advan-

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tages, the complicated procedures, use of highly sophisticated equipment, or lengthy execution of experiments and other limitations prevent widespread measurement of this important biological process.

In order to develop a rapid and reliable assay to measure gap-junctional communication, we extended an observation by McNeil et al. [27], whereby certain non-permeable molecules could be introduced into cells via a 'scrape-loading' technique. Scrape-loading has been effectively utilized to introduce macromolecules into cells in culture by inducing a transient tear in the plasma membrane without affecting cell viability or colony-forming ability [27]. The tracer dye Lucifer yellow (MW 457.2) is an intensely fluorescent 4-aminophthalimide with a high quantum yield of about 0.25 [28, 29]. This yield is stable between pH 1 and 10 and allows its detection with epifluorescence microscopy at low, non-cytotoxic concentrations. Lucifer yellow does not diffuse through intact plasma membranes and its low MW permits its transmission from one cell to another, presumably across patent gap junctions [28–30]. Preliminary experiments with the high MW dye conjugate, rhodamine dextran (MW 10000), indicated that it can neither diffuse through intact plasma membranes nor cross the junctional channels. Upon excitation, rhodamine dextran emits red fluorescence with a spectrum distinct from that of Lucifer yellow. The concurrent introduction of both Lucifer yellow and rhodamine dextran into cells allows the identification of the primary loaded cells and therefore verifies that Lucifer yellow transfer to contiguous cells occurs through membrane junctions.

We report here experiments indicating that this scrape-loading/dye transfer technique can be used to detect gap junctional communication in a wide variety of mammalian cells grown in vitro.

MATERIALS AND METHODS

To test the efficiency of the technique in detecting intercellular communication, five different lines and three primary cultures of mammalian cells were utilized. Chinese hamster V79 cells, rat glial primary cell culture derived from the cerebral tissue of a 20th day gestation rat fetus [31], rat glioma cells, WB rat liver cells, human teratocarcinoma [32], and the primary culture of human foreskin fibroblasts (MSU-2) were grown to confluency on 35 mm plastic plates in modified Eagle's medium with Earle's balanced salt solution with a 50% increase in vitamins and essential amino acids, except glutamine. The medium was supplemented with a 100% increase in non-essential amino acids, 1 mM sodium pyruvate and 3–10% fetal calf serum (FCS) depending on the cell type. The cells were incubated at 37°C in humidified air with 5% CO_2. A primary culture of calf aorta muscle cells obtained from tissue explants [33] was synchronized in defined, serum-free media (1:1 F12 and Dulbecco supplemented with insulin 10^{-6} M, transferrin 5 μg/l and ascorbic acid 0.5 M) [34], and incubated at similar conditions. NIH/3T3 mouse fibroblasts were cultured in Dulbecco's modified MEM media supplemented with 10% FCS at 37°C in humidified air containing 8% CO_2.

Cells were rinsed with PBS before the addition of the fluorescent dye mixture. Two milliliters of 0.05% Lucifer yellow and rhodamine dextran (purchased from Molecular Probes, Inc, Eugene, Oreg.) dissolved in PBS were added to the cells and scrape-loaded at room temperature using a rubber policeman or wooden probe. The dye solution was left on the cells for 2 min, then discarded and the plates rinsed with PBS to remove detached cells and background fluorescence. Two milliliters of media were replaced and cells were examined under a Nikon epifluorescene phase microscope illuminated with an Osram HBO 200 W lamp. Control plates were set by exposing the cells under similar

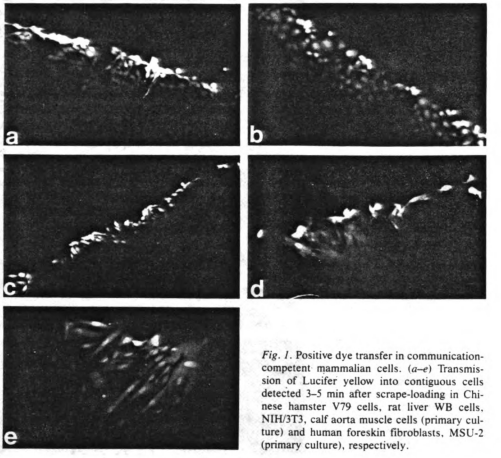

Fig. 1. Positive dye transfer in communication-competent mammalian cells. (*a–e*) Transmission of Lucifer yellow into contiguous cells detected 3–5 min after scrape-loading in Chinese hamster V79 cells, rat liver WB cells, NIH/3T3, calf aorta muscle cells (primary culture) and human foreskin fibroblasts, MSU-2 (primary culture), respectively.

conditions to the dye mixture but without scraping. The cells were then examined for fluorescence after rinsing with PBS. To determine the sensitivity of the assay in detecting agents that block intercellular communication, the cells were exposed to a series of known tumor promotors of variable potency at non-cytotoxic doses. The chemicals used included the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA), dieldrin, teleocidin, saccharin and mezerine, in addition to a negative control, 4-phorbol-12, 13-didecanoate (4α-PDD). Cells were treated with each chemical for an exposure time predetermined by previous studies [10, 11, 35–39].

RESULTS

In control experiments, cells treated with the dye mixture without scrape-loading did not pick up either dye after an equivalent exposure time. All cells scrape-loaded in the presence of a mixture of Lucifer yellow and rhodamine dextran showed a positive transfer of the Lucifer yellow alone into contiguous cells (fig. 1). This dye transmission occurred shortly after loading. The marker dye rhodamine dextran remained entrapped, thus labelling the primary loaded

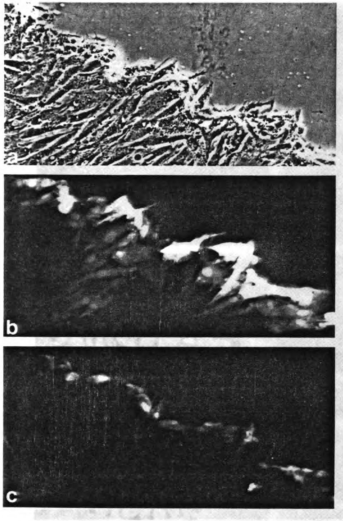

Fig. 2. Identification of primary loaded cells. (*a*) Phase-contrast photomicrograph of human foreskin fibroblasts (MSU-2) after scrape-loading in the presence of both Lucifer yellow and rhodamine dextran dyes; (*b*) positive transmission of Lucifer yellow into contacting cells; (*c*) rhodamine dextran shown confined to the primary loaded cells. (*a*, *b*, *c*) Photomicrographs representing the same scraped area.

cells at the edge of the scraped areas (fig. 2). There was also a noticeable Lucifer yellow fluorescence gradient, with its highest intensity in cells at the periphery of the scraped areas.

Making the assumption that there was no differential effect of scrape-loading on gap-junction function between cell types, the various cells examined differed in their ability to communicate as measured by the extent of the dye transfer. Chinese hamster V79 and mouse NIH/3T3 cells were the least efficient, whereas rat glial cells, calf aorta muscle cells, and human foreskin fibroblasts (MSU-2), all primary cultures, showed extensive spread of fluorescence within the same period of time. In most cells, the decrease in Lucifer yellow fluorescence intensity over time was accompanied by its further spread into contiguous cells. In all

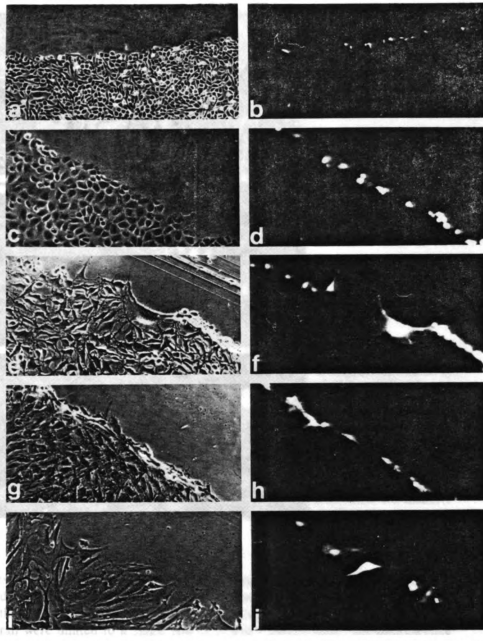

Fig. 3. **Blockage of dye transfer by tumor promoters. Non-cytotoxic doses of the phorbol ester TPA (5 and 10 ng/ml), dieldrin (7 μg/ml), teleocidin (1 ng/ml), saccharin (5 mg/ml), mezerine (2 ng/ml) and the negative control 4α-PDD (1 ng/ml) were each added separately to cells in culture prior to scrape-loading for a period of time that ranged from 15 min to 8 h (data on teleocidin, saccharin, mezerine and 4-PDD not shown). Effective blockage of dye transmission was only observed in cells pretreated with tumor promoters. (*a, b*) V79 cells treated with TPA (5 ng/ml) for 15 min; (*c, d*) rat liver cells (WB) after 3 h of dieldrin (7 μg/ml); (*e, f*) NIH/3T3 cells pretreated with TPA (5 ng/ml) for 1 h (*g, h*) calf aorta muscle cells (primary culture) after 30 min exposure to TPA (10 ng/ml); (*i, j*) human foreskin fibroblasts (MSU-2) following 1 h in TPA (5 ng/ml).**

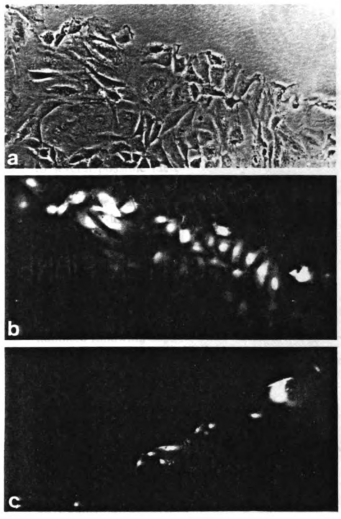

Fig. 4. Blocking of effective intercellular communication in rat glial primary culture by the neurotoxin dieldrin. (*a*) Cells viewed with phase-contrast microscopy after scrape-loading; (*b*) positive cell–cell communication is shown by Lucifer yellow transfer in contiguous cells; (*c*) inhibition of Lucifer yellow transmission following pretreatment with dieldrin (7 μg/ml) for 3 h.

plates treated with tumor promoters, including teleocidin, saccharin and mezerine (data not shown), the cells appeared to have lost their ability to communicate and the dye transfer was blocked (figs 3, 4). Both Lucifer yellow and rhodamine dextran were limited to a single row of primary loaded cells. The fluorescence remained intense for hours after loading with no indication of further dye transfer of leakage. In cells treated with the negative control 4α-PDD no inhibition of Lucifer yellow transmission was observed (data not shown). Regeneration and proliferation of primary loaded and secondary recipient cells were observed 24 h following scrape-loading, indicating that the procedure has no apparent adverse effect on cell viability.

DISCUSSION

The results obtained using the scrape-loading and dye transfer technique are consistent with previous studies [10, 11, 35–39]. Cells, shown by other techniques to have gap-junctional communication, were also shown here to have a gap junction-dependent transfer of dye after scrape-loading. Known tumor promoters inhibit this scrape-loading/dye transfer process in a way similar to that shown by other more complicated methods which, themselves, have been shown to be comparable in measuring gap-junctional communication [40]. The observation that different cell types varied in their ability to communicate might be attributed to several possible factors including cell type, tissue of origin, number of pre-existing and functional membrane junctions, mitotic activity, differential reaction to the scrape-loading process, cell volume and state of transformation or differentiation. The decrease in fluorescence with time in communication-competent cells is apparently the result of further dye spreading across membrane junctions. This observation was not made in cells blocked by tumor promoters.

Among the tested chemicals the scrape-loading technique was used to study the effect of dieldrin, a known tumor promoter and neurotoxin [41, 42] on a primary culture of fetal rat glial cells. The remarkable communication demonstrated by the extent of Lucifer yellow transfer in untreated cells was strongly inhibited following exposure to a non-cytotoxic dose of dieldrin (7 μg/ml) prior to scrape-loading (fig. 4). This is consistent with the observations that dieldrin inhibited gap junction in human teratocarcinoma cells using metabolic cooperation [39] and FRAP [26] techniques. The detection of this chemically induced interference with junctional communication in cells derived from brain tissue may carry important research implications in that it tends to support the observation that many tumor promoters can be neurotoxins [17] and that cell–cell communication may play an important role in brain development and function [43, 44]. The described technique might allow the study of cell–cell interaction in various cells derived from the brain and its modulation following exposure to drugs or other environmental factors known or suspected to affect brain function. Further investigations are needed to substantiate the close correlation between the presence of gap junctions and the occurrence of electrotonic and dye coupling in the brain tissue of various animals [43, 44].

The described technique provides a direct and low cost approach to study gap junctional intercellular communication in cultured cells with the results obtained in a few minutes. It can be applied to a wide variety of cells grown in monolayer to study potential inhibitors of junctional communication and explore aspects of their mechanism of action. The follow-up of the same cells after reincubation allows the modification of dose, exposure and recovery time, as well as other experimental parameters. The rapidity of the assay ensures a specific and instant testing of cell–cell communication among secondary dye-recipient cells. It thus minimizes physiological alterations or artifacts that may be caused by other lengthy and complicated procedures. The technique offers sensitive and remark-

able qualitative determination of cell–cell communication. For quantitative evaluation, the extent of the dye transfer can be estimated by counting the number of fluorescing secondary recipient cells in randomly selected areas on the plate. The possibility exists for automated quantitation by combining this technique with computerized FRAP analysis [26]. Our preliminary studies have demonstrated that dose–response effects can be observed and measured by both counting the fluorescing secondary recipient cells and FRAP analysis (M. G. Evans & M. H. El-Fouly, unpublished data). A further advantage of the method lies within its ability to monitor intercellular communication *among* as well as *between* different cell types grown in co-culture. The application of the technique to cells in vitro may prove valuable in testing the hypothesis that the inhibition of intercellular communication is involved in the promotion phase of carcinogenesis. It may also be developed as a rapid assay to screen for suspected chemical modulators of gap junctional communication and to investigate their effects on tissue development and cellular interactions.

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Dieldrin Inhibition of Gap Junctional Intercellular Communication in Rat Glial Cells as Measured by the Fluorescence Photobleaching and Scrape Loading/Dye Transfer Assays[1]

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Dieldrin Inhibition of Gap Junctional Intercellular Communication in Rat Glial Cells as Measured by the Fluorescence Photobleaching and Scrape Loading/Dye Transfer Assays. SUTER, S., TROSKO, J. E., EL-FOULY, M. H., LOCKWOOD, L. R., AND KOESTNER, A. (1987). *Fundam. Appl. Toxicol.* **9**, 785-794. Application of the fluorescence-recovery after photobleaching (FRAP analysis) technique and scrape loading/dye transfer assay was made to measure the presence of gap junctional communication in primary rat glial cells *in vitro* in the presence and absence of the neurotoxicant and tumor promoter dieldrin, a chlorinated insecticide. Results demonstrate that primary rat glial cells are able to exhibit gap junctional intercellular communication and that dieldrin at noncytotoxic concentrations can modulate gap junctional communication as early as 10 min after exposure to the chemical and that the effect is reversible after 4 hr recovery from the dieldrin exposure. Both the FRAP analysis and the scrape loading/dye transfer assay have validated the observation that dieldrin inhibits gap junctional communication in other cell types using different techniques to measure gap junction function. These results were interpreted as an indication that inhibition of gap junctional communication might contribute to the cellular mechanism of dieldrin's neurotoxicity. © 1987 Society of Toxicology.

Gap junctional-mediated intercellular communication has been regarded as an important determinant for homeostasis in organisms composed of functionally specialized cells for normal cell growth and differentiation, reproductive, neuroendocrine, and cardiac function, and a whole host of other normal physiological states (Bennett and Goodenough, 1978; Loewenstein, 1979; Hertzberg *et al.*, 1981; Pitts, 1980; Bennett *et al.*, 1981; Schultz, 1985; Larsen, 1983). Low-molecular-weight substances (≤1500 MW) can be transported from cell to cell via gap junctions on contiguous cells (Loewenstein, 1979). Disruption of gap junctional intercellular communication has been postulated to play a role in carcinogenesis (Loewenstein and Kanno, 1966), specifically during the tumor-promotion phases (Yotti *et al.*, 1979; Murray and Fitzgerald, 1979; Trosko *et al.*, 1983). In addition, many tumor-promoting chemicals (Jone *et al.*, 1985) and a few oncogenes (Chang *et al.*, 1985; Azarnia and Loewenstein, 1984; Atkinson and Sheridan, 1984; Atkinson *et al.*, 1986; Azarnia and Loewenstein, 1987) have been associated with inhibited intercellular communication.

Gap junctional intercellular communication has been measured by a variety of techniques, including electrocoupling (Furshpan

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and Potter, 1959); intercellular transfer of injected fluorescent dyes (Loewenstein, 1966); use of genetically deficient cells to measure "metabolic cooperation" (Hooper, 1982; Davidson *et al.*, 1985; Gupta *et al.*, 1985); and autoradiographic detection of the transfer of low-molecular-weight radioactive labeled compounds (Subak-Sharpe *et al.*, 1969). The ultrastructural analysis of gap junctions is performed by freeze–fracture analysis of cell membranes (Finbow and Yancey, 1981; Larsen, 1983; Larsen and Risinger, 1985). Recently, two new techniques, one using fluorescence-recovery after photobleaching (FRAP analysis), and the other, the scrape loading/dye transfer assay, have been applied to measure gap junctional intercellular communication (Wade *et al.*, 1986; El-Fouly *et al.*, 1987).

Dieldrin, belonging to the cyclodiene class of chlorinated insecticides, is a well-documented toxic chemical. It has been found to be carcinogenic in laboratory rodents, specifically it seems to act as a tumor promoter (Ito *et al.*, 1980; Tennekes *et al.*, 1982). Similar to 12-tetradecanyolphorbol-13-acetate (TPA), dieldrin has been shown to be nonmutagenic in most genotoxic assays (McCann *et al.*, 1975; Ashwood-Smith, 1981; Purchase *et al.*, 1978; Probst *et al.*, 1981; Tong *et al.*, 1981; ICPEMC, 1984). On the other hand, dieldrin has been shown to inhibit metabolic cooperation (a form of gap junctional communication) in Chinese hamster V79 cells (Trosko *et al.*, 1987) and human teratocarcinoma cells (Lin *et al.*, 1986). In addition, dieldrin is known to be a neurotoxin (Joy, 1982). Since gap junctions are known to exist in neuroectodermal cells, this study was designed to determine if some of the neurotoxic effects of dieldrin might be related to its ability to inhibit gap junctional intercellular communication.

MATERIALS AND METHODS

Cells. Normal rat glial cells were subcultured from primarily cultured rat glial cells isolated from cerebral tissue of rat fetuses at the 20th gestation day (Ko *et al.*, 1980). Cells within 10 passages were grown in modified Eagle's medium (MEM; GIBCO formulas 78-5470; Earle's balanced salt solution with 50% increase of vitamins and essential amino acids except glutamine), supplemented with nonessential amino acid (100% increase), 1 mM sodium pyruvate, and 10% fetal calf serum. Under the incubation condition with 5% CO_2 in humidified air at 37°C, cells growing in monolayer, contact-inhibited upon confluency, were subcultured every 5 to 7 days.

Chemicals. 5 (and 6)-Carboxyfluorescein diacetate and rhodamine lissamine dextran (Lot 5B) were obtained from Molecular Probes (Eugene, OR). Lucifer yellow CH was from Sigma Chemical Co. (St. Louis, MO). Dieldrin [Shell Chemical Co. (purity 99+%)] was a gift from Dr. B. V. Madhukar of the Pesticide Research Center at Michigan State University.

Methods. Experiments were performed with rat glial cells plated in the modified MEM. Dieldrin, dissolved in ethyl alcohol (ETOH), was added to cells for various lengths of time to give a final concentration of 7 μg/ml of medium (0.1% final concentration of ETOH). An identical volume of ethyl alcohol, the solvent carrier, was added to the control cells. Neither the solvent carrier nor the carrier plus dieldrin was cytotoxic to the cells at this concentration nor did the concentration of ETOH interfere with intercellular communication.

To measure gap junctional communication using the FRAP analysis technique, following 24 hr of growth, the cells were washed with PBS containing calcium (0.9 mM) and magnesium (0.5 mM; PBS/Ca/Mg) and stained with 6-carboxyfluorescein diacetate. The dye and labeling conditions do not affect cell viability, and restaining can be performed on the same cells for several days. All measurements are performed at room temperature in PBS/Ca^{2+}/Mg^{2+} within a 1-hr period. A tissue culture plate of labeled cells is placed on a high-speed computer-controled two-dimensional stage of the ACAS 470 workstation (Wade *et al.*, 1986). The Meridian ACAS 470 (Anchored Cell Analysis and Sorting, Meridian Instruments, Okemos, MI) was the standard instrument which was equipped with a 2-W argon ion laser tuned to the 488-nm line, dichroic filter at 510 nm and barrier filter at 520 nm, inverted phase-contrast microscope and 16-bit microcomputer for data acquisition and processing, and micro-stepping stage. The stage moves the cells in a defined manner above the objective (40×) of an inverted epifluorescence microscope. The microscope objective serves to focus the argon laser beam (excitation wave length of 488 nm) to a 1-μm spot size that excites fluorescence in individual cells at 1.5-μm steps in a two-dimensional raster pattern. The single-point emission from each excited step is recorded as an intensity by a photomultiplier tube. The digital signals representative of fluorescence intensity are stored in the computer with the source x–y location. The emitted intensities are color

coded and presented on a computer video screen as a pseudo-color image of the fluorescence distribution in the analyzed cell.

In order to measure gap junctional communication by another independent method, the scrape loading/dye transfer assay was utilized. Rat glial cells were subcultured using trypsin (0.01%) without EDTA and plated to attain a confluent monolayer ($1.5–2.0 \times 10^6$ cells) in 35-mm plastic dishes. The cells were incubated in the modified Eagle's medium with 5% FCS at 37°C in humidified air with 5% CO_2 for 12–18 hr. Six plates were prepared for each experimental point including untreated controls and controls with solvent (0.1% absolute ethanol final concentration) only. For temporal studies, the cells were treated with a single dose of dieldrin for various exposure times (6, 10, 15, 20, 30, 50, and 60 min and 24 hr). This predetermined noncytotoxic dose of 7 μg/ml has been previously shown to induce a complete blockage of gap junctional intercellular communication and dye transfer in rat glial cells (El-Fouly *et al.*, 1987). In addition, to test for the reversibility of the dieldrin effect on gap junction conductance following a short-term exposure, the cells were treated with dieldrin (7 μg/ml) for 1 hr then washed with PBS and reincubated after the addition of fresh media for 24 hr.

For dose–response experiments, dieldrin was added to each plate at various noncytotoxic concentrations (1, 2, 3, 5, 6, 7, and 10 μg/ml) for a fixed 2-hr exposure time prior to scrape loading.

In preparation for scrape loading/dye transfer, the cells were washed with PBS (kept at room temperature), then exposed to a dye mixture containing 0.05% of each of Lucifer yellow (MW 457.2) and rhodamine lissamine dextran (MW 10,000) dissolved in PBS. The dye molecules were loaded intracellularly by scraping or cutting the cells using a wooden probe or a sharp knife. The dye solution was left on the cells for 90 sec, then discarded, and the plates were carefully rinsed in PBS to minimize the background fluorescence. The cells were next examined for dye transfer under an inverted Nikon epifluorescence phase microscope with uv light generated from an Osram HBO 200-W bulb. The degree of communication was assessed by measuring the extent of Lucifer yellow transfer into contiguous cells. Quantitation was estimated by counting the number of secondary recipient cells in a fixed surface area selected at random. Ten different fields were examined per plate, six plates per treatment, and an average count is reported as a relative percentage compared to control plates which were considered to have 100% communication.

RESULTS

The effect of dieldrin on the colony-forming ability is shown in Fig. 1. Results show

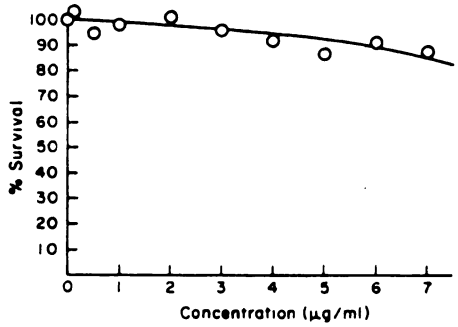
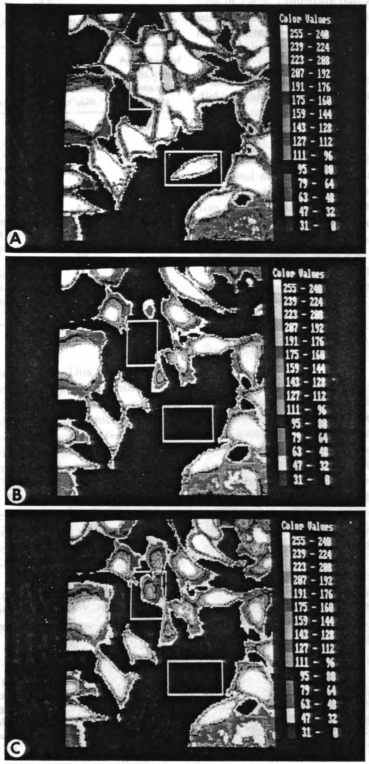


FIG. 1. Effect of dieldrin on the colony-forming ability of primary rat glial cells. The plating efficiency was 87%. Five plates per dose level were counted.

that after a 3-day exposure, even at the highest concentration (7 μg/ml), very little effect was noted in terms of inhibition of the plating efficiency and formation of colonies. It must be noted that the cytotoxicity assay is performed at very low cell densities (200 cells/60-mm plate), and long exposures to the chemical (3 days), whereas the effect of dieldrin on gap junctional communication is done on high densities of cells for short periods of time. Therefore, these cytotoxicity data would be considered overestimates of the effective cytotoxic levels. In other words, dieldrin at 7 μg/ml (or up to 10 μg/ml) for scrape loading/dye transfer) should not be cytotoxic under the conditions used to measure its effect on gap junctional communication.

In order to ascertain whether FRAP analysis could detect gap junctional intercellular communication in primary rat glial cells, an experiment, as illustrated in Fig. 2, was performed. The results clearly demonstrate that 18 min after photobleaching of a single untreated cell, the fluorescence reappeared in coupled cells, but not in isolated cells. This is interpreted as indicating that the carboxyfluorescence dye was transferred, via gap junctions, to the photobleached cell. The lack of fluorescence in the isolated cell demonstrates that a new source of dye can be replaced only from gap junctionally coupled


Color Values
255 - 240
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223 - 208
207 - 192
191 - 176
175 - 160
159 - 144
143 - 128
127 - 112
111 - 96
95 - 80
79 - 64
63 - 48
47 - 32
31 - 0
A
B
C

TABLE 1

RESULTS OF FRAP ANALYSIS ON DIELDRIN-TREATED RAT GLIAL CELLS

Treatment	Number of cells	Percentage of cells with fluorescence recovery
Control	25	100
24 hr pretreatment with dieldrin (7 μg/ml)	40	0.0
1 hr pretreatment with dieldrin (7 μg/ml)	6	0.0
10 min pretreatment with dieldrin (7 μg/ml)	43	0.0
1 hr pretreatment with dieldrin plus 4 hr post-treatment minus dieldrin	20	100
1 hr pretreatment with dieldrin plus 1 hr post-treatment minus dieldrin	6	83

unphotobleached cells. This figure is typical of 25 other samples in the same dish.

To test if dieldrin could inhibit gap junctional intercellular communication in rat glial cells, the experiment showed that 24 hr treatment with 7 μg/ml dieldrin prevented gap junction-mediated transfer of the fluorescent dye (Table 1).

Results in Table 1 also demonstrate that a 1-hr treatment of 7 μg/ml dieldrin was sufficient to inhibit gap junctional communication as measured by FRAP analysis. This result was typical of six randomly chosen coupled cells.

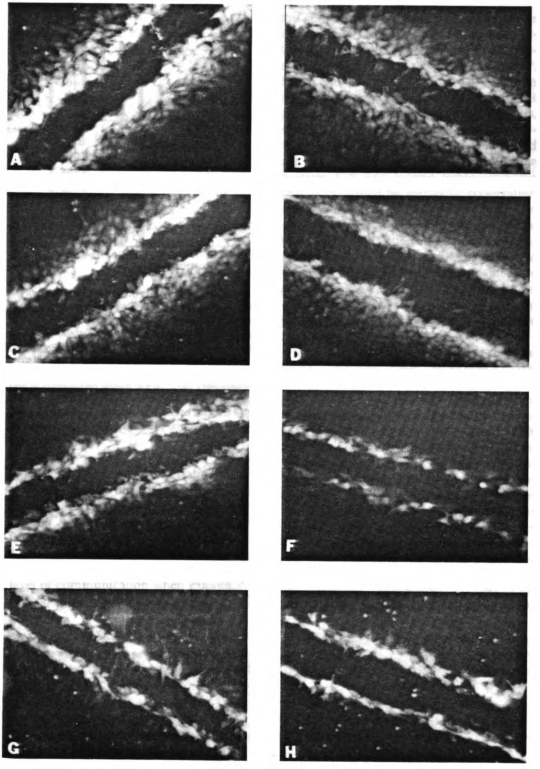
To determine if shorter treatment times could inhibit gap junctional communication a series of experiments were performed. The data in Table 1 illustrate that a 10-min exposure to 7 μg/ml dieldrin was sufficient to inhibit communication. Again, this result was repeated in 43 other randomly chosen coupled cells.

Since it is important to determine if the dieldrin inhibition of intercellular communication is either irreversible or reversible, cells were treated with dieldrin (7 μg/ml) for 1 hr and allowed to "recover" for 1 and 4 hr after the dieldrin was removed. Cells were washed twice and placed in fresh non-dieldrin containing medium. Data in Table 1, representative of *20* random samples, show that a 4-hr post-treatment time was sufficient for the reestablishment of gap junctional communication. In addition, results also indicate that 1 hr seems sufficient for these rat glial cells to reestablish gap junctional communication in six randomly chosen cells.

Scrape Loading Results

The results obtained from scrape loading/dye transfer assay are shown in Figs. 3 and 4. Quantitative analysis of the dose–response data, as described under Materials and Methods, indicates a direct correlation between the extent of blockage of gap junctional transfer of Lucifer yellow and the dieldrin concentration applied for a fixed period of 2 hr (Figs. 3 and 4A). When the cells were treated for variable exposure times with a fixed dose of dieldrin (7 μg/ml), the extent of junctional communication was inversly correlated with the duration of treatment (Fig. 4B; also data not shown). Complete inhibition of dye transfer was observed after approximately 50 min of initiating the treatment (Fig. 4B). The

FIG. 2. Restoration of fluorescence in photobleached, control primary rat glial cells. By comparing the images generated before photobleaching, when all cells were highly fluorescent as indicated by the false-color image in (A), with images produced 1 min (B) and 18 min (C) after bleaching, the recovery of fluorescence could be monitored. The image in (C) clearly shows the contacting, but not the isolated, cell regained its image after 18 min postbleaching. Image is ×300.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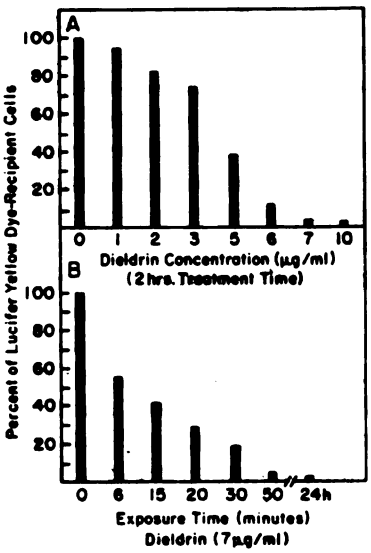

FIG. 4. (A) Inhibition of dye transfer in rat glial cells by dieldrin as detected by scrape loading/dye transfer assay. The method for quantitation is described under Materials and Methods. Dose-response effect of dieldrin on gap junction-mediated Lucifer yellow transfer. The cells were treated with variable concentrations of dieldrin for a fixed period of 2 hr. Panel (A) is a graphic representation of experiments shown in Fig. 3. (B) Time-course of the effect of dieldrin on dye transfer. A single treatment dose of dieldrin (7 μg/ml) was added to the cells for the indicated time periods followed by scrape loading of Lucifer yellow.

blockage of cell–cell communication by a single application of dieldrin was sustained for over 24 hr (Fig. 3). The inhibition of dye transfer was reversed when the cells were transiently exposed to dieldrin (7 μg/ml) for 1 hr then released and re-incubated in fresh medium. These cells resumed their control level of communication when examined 24 hr following the removal of dieldrin (data not shown).

DISCUSSION

There seem to be several conclusions resulting from the observations made during this study: (a) FRAP analysis and the scrape loading/dye transfer assay have verified earlier conclusions that gap junctional communication exists in rat glial cells (Orkand, 1977; Massa and Mugnaini, 1985); (b) dieldrin can inhibit gap junctional communication in rat glial cells, as measured by FRAP analysis, supporting previous observations that noncytotoxic levels of this toxic chemical inhibited gap junctional communication in Chinese hamster V79 and human teratocarcinoma cells as measured by metabolic cooperation and uridine transfer (Trosko *et al.*, 1987; Lin *et al.*, 1986); (c) FRAP analysis and scrape loading/dye transfer techniques, by corroborating the aforementioned studies, seem to be validated as a legitimate means to measure gap junctional intercellular communication; and (d) the effect of dieldrin inhibition of gap junctional communication is a reversible phenomenon.

Since gap junctional intercellular communication has been postulated to play a major role in the regulation of development, cell proliferation, regeneration, differentiation, homeostasis, and control of differentiated cell functions (Loewenstein, 1979; Pitts, 1980; Hertzberg *et al.*, 1981; Schultz, 1985; Larsen, 1983) in multicellular organisms, it seems logical to conclude that exogenous and endogenous chemical modulation of gap junction structure and/or function would have adaptive and nonadaptive consequences (Trosko and Chang, 1984). Many chemicals, which are known to be tumor promoters, have been demonstrated to be inhibitors of gap junctional communication (Jone *et al.*, 1985; Trosko *et al.*, 1982; Malcolm *et al.*, 1985).

One of those chemicals which is a tumor promoter of rat liver tumors and which inhibits gap junctional communication is dieldrin.

FIG. 3. Dose–response effect of dieldrin on junctional permeability in rat glial cells as measured by the scrape loading/dye transfer technique. The photomicrographs show Lucifer yellow transfer into contiguous cells pretreated with various concentrations of dieldrin. (A) Untreated control cells; (B–H) cells pretreated for 2 hr with 1, 2, 3, 5, 6, 7, and 10 μg dieldrin/ml, respectively.

What makes the dieldrin effect on the inhibition of gap junctional communication in rat glial cells relevant to these results is that dieldrin is also a known neurotoxin (Joy, 1982). In the former case, it has been postulated that when gap junctional communication is inhibited in tissues where a single carcinogen-initiated stem cell is repressed by surrounding normal cells, the initiated cell then clonally expands to form a tumor (Yotti *et al.*, 1979; Trosko *et al.*, 1983). In the latter case, although the role of gap junctional communication in neural cells has not been as well studied as the chemical neurotransmission form of intercellular communication, it is known to exist in brain tissue (Andrew *et al.*, 1981). In addition, two neurotransmitters, acetycholine and dopamine, have been shown to modulate gap junction function from several organisms (Iwatsuki and Petersen, 1978; Findlay and Petersen, 1982; Teranishi *et al.*, 1983; Piccolino *et al.*, 1984; Lasater and Dowling, 1985; Neyton and Trautmann, 1986). Since it would be hard to imagine, in evolutionary terms, that gap junctional communication plays no role in this highly specialized tissue, modulation of gap junction function in brain cells by dieldrin might be expected to play some role in its neurotoxicity.

Finally, as a note of speculation, one could imagine that, in the brain, chemical neurotransmission and gap junction transfer of ions and small molecular weight molecules comprise a highly coordinated and integrated intercellular communication network (Bennett *et al.*, 1985). Conceivably, gap junctional communication provides a means to regulate growth control and differentiation of premitotic cells, as well as a means to provide "nutrients" and regulatory signals to postmitotic neural cells. Endogenous and exogenous modulation of gap junctional communication in either pre- or postmitotic brain cells could have both adaptive, as well as toxic, consequences, depending on the nature of the inhibition.

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DISCUSSION

The data obtained from the reported studies verified the fact that the wild type Chinese hamster fibroblasts (V79) have competent gap junctional intercellular communication. This assessment was determined by both the metabolic cooperation and the scrape-loading/dye transfer assays. These cells were stably transformed following transfection with recombinant plasmids: the control pSV_2neo, containing the neomycin resistance gene as a selective marker, and the $pSWO_4$ plasmid. The latter contains both the selective marker and the entire human c-Ha-ras-1 oncogene derived from the EJ/T4 bladder carcinoma (Shih and Weinberg 1982). Southern blot analysis showed the presence of a faint 6.6 Kb band in the wild type control cells and more intense bands of similar and variable sizes in all BamHI-digested DNA from clones transfected with $pSWO_4$, indicating variable integration sites (Figure 4). The stability of transformation was verified, and maintained, by selection in G418 antibiotic.

The expression of c-Ha-ras-1 was tested by indirect immunofluorescence using monoclonal antibody, clone Y13-259 (Furth et al 1982). Intense fluorescence was detected in those cells transfected with c-Ha-ras-1, as compared to a barely detectable trace in the wild type cells and in cells transfected with the control plasmid pSV_2neo (Figure 5). The cells expressing the p21 ras protein acquired a more transformed morphology as compared to control wild type and those transfected with pSV_2neo alone. The alterations included spindle-shape morphology,

refractile cell outline with an irregular growth pattern and lack of contact inhibition. The cells appeared overlapping and piling in clumps with necrotic centers. This was in addition to a marked decrease of cell adherence to the substrate and, in some cells, the appearance of cytoplasmic vacuoles.

In the metabolic cooperation assays, the variety of the co-cultured combinations resulted in a remarkable recovery of $6TG^r$ colonies whenever the c-Ha-ras-1 oncogene was expressed in either or both co-cultured cell types. Statistical analysis of three different experiments of metabolic cooperation showed reproducible results ($p < 0.0005$ to $p < 0.00001$). The results obtained by assaying for gap junctional communication utilizing the scrape-loading/dye transfer technique were in agreement with those of the metabolic cooperation assays. All cells transfected with c-Ha-ras-1 showed complete inhibition of Lucifer yellow dye transfer as compared to the wild type and pSV_2neo transfected control cells. In the control series, the homogeneity of gap junctional communication ability among the pooled wild type cells was verified in eighteen different subclones by scrape-loading/dye transfer and six of them were reported in two of the metabolic cooperation assays performed.

From the data obtained in the series of experiments reported here, a strong correlation appears to exist between the expression of the c-Ha-ras-1 product i.e., the p21 polypeptide, as detected by monoclonal antibody against p21, and the inhibition of cell-cell communication via the membrane gap junctions. This correlation resembles those effects exerted by exogenous chemical tumor promoters (Yotti et al 1979, Murray and Fitzgerald 1979, Trosko et al 1987), by certain growth factors

(Madhukar et al 1988), metabolites (Malcolm et al 1985), hormones (Merk et al 1972, Decker 1976, 1981, Dahl and Berger 1978, Garfield et al 1980), and by membrane bound oncogenic products (Azarnia and Loewenstein 1984b, Chang et al 1985). This end point parallelism between the p21 effects and those of factors that modulate gap junctional intercellular communication lend support to the hypothesis under study, i.e. that the oncogenic product of the H-ras may play a role in the process of tumor promotion by inhibition of junctional coupling.

The symmetry between the endogenous expression of ras p21 and the exogenous exposure to tumor promoters in modulating cellular gap junctions has its grounds in several remarkable properties of the ras gene product. The oncogenic, as well as the normal cellular ras proto-oncogenes, products share a common intracellular translocation site, i.e. the inner side of the plasma membrane (Willumsen et al 1984), and they only differ by a single point mutation at specific nucleotides (Barbacid 1987). Both the oncogene and the proto-oncogene have structural, as well as biochemical similarities to the G-proteins which are components of two major membrane signal transduction systems, the adenylate cyclase (Wakelam et al 1986) and PIP_2 (Scolnick et al 1979, Willingham et al 1980). The G-proteins, also known as guanine-nucleotide-binding proteins, are coupled to cell surface receptors and assist in the process of mediating a mitogenic signal to an effector molecule(s) via modulating adenylate cyclase. This modulation alters the intracellular levels of cAMP which, when elevated enhance gap junction-mediated communication (Saez et al 1986, Wiener and Loewenstein 1983, Johnson et al 1985). Activation of G-proteins subsequently lead to intracellular signal transduction (Sweet et al 1984, McGrath et al

1984, Lacal et al 1986, Gibbs et al 1984, Colby et al 1986). Recently, ras expression was found to correlate with decreased adenylate cyclase activity and low cAMP levels in NIH3T3 cells (Hiwasa and Sakiyama 1986). Given that low cAMP is associated with decreased gap junctional communication (Saez et al 1986, Wiener and Loewenstein 1983, Johnson et al 1985), those observations do not contradict the results obtained here, i.e. that ras expression is accompanied by decreased cellular coupling.

The ras product was found to be an integral component of the very versatile membrane signaling pathway, the inositol phosphate system (Fleishman et al 1986). This pathway transmits signals induced by growth factors and other mitogenic agents intracellularly, which induce an action that results in DNA synthesis and cell proliferation (Feramisco et al 1984, Stacey and Kung 1984). In this system (see Figure 14), the membrane phosphoinositides (PIP) are cleaved by phospholipase C, which could be activated by the G-proteins, into two byproducts, the diacylglycerol (DAG) and inositol triphosphate (IP3) (Berridge 1983). The diacylglycerol is an essential co-factor for PKC activation (Nishizuka 1984) and the IP3 is known to mobilize the calcium ions from intracellular, non mitochondrial stores (Berridge et al 1984). The H-ras expression was reported to stimulate an increased production of DAG, as well as inositol phosphates in ras transformed cells (Lacal et al 1987, Wolfman and Macara 1987, Chiarugi et al 1986, Preiss et al 1986, Fleischman et al 1986, Wakelam et al 1986). The DAG is known to activate PKC (Berridge 1984, Kikkawa et al 1983, Sharkey et al 1984) which also acts as a receptor to, and is activated by, the potent tumor promoter, TPA (Castagna et al 1982, Niedel et al 1983,

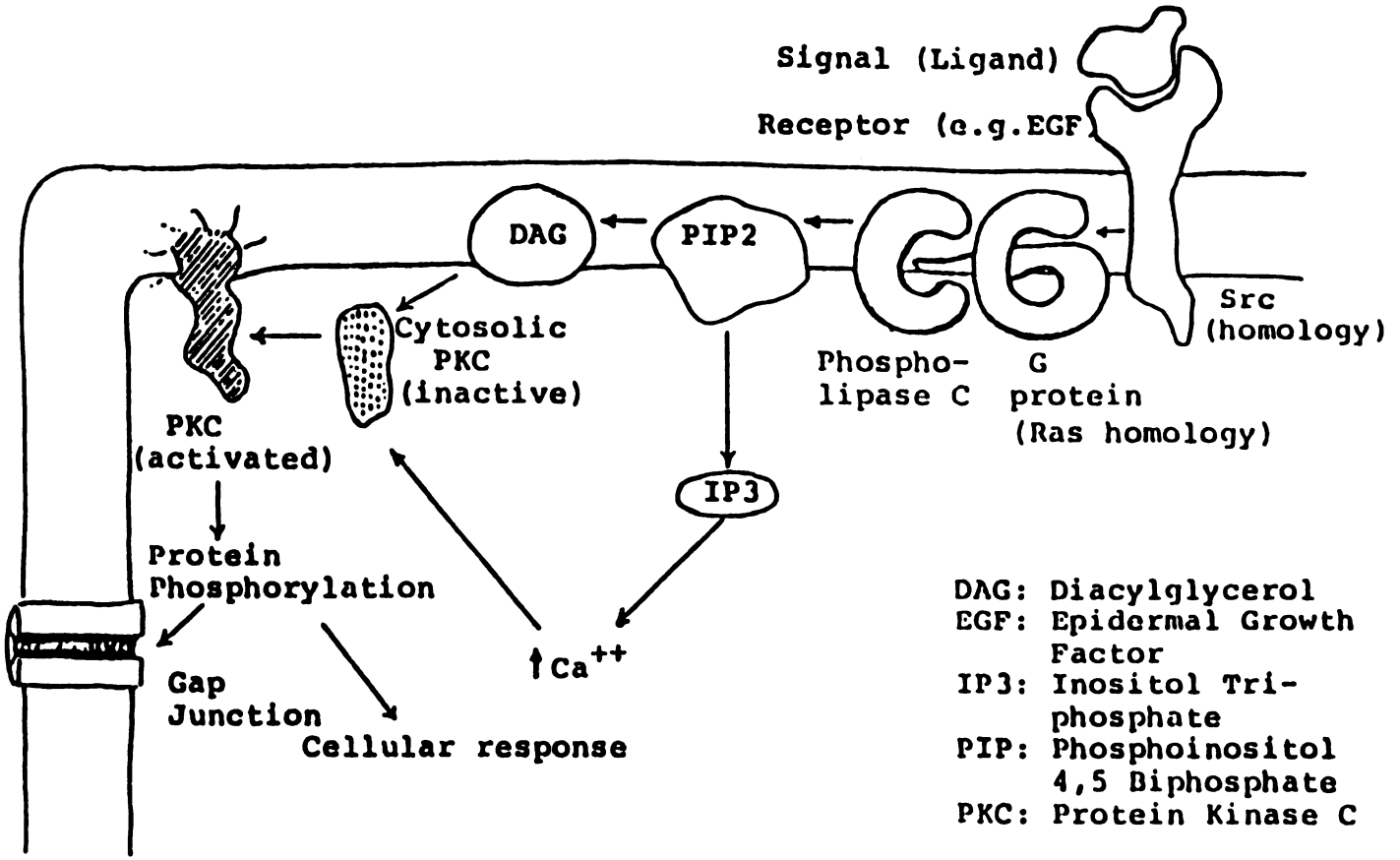

Figure14. Schematic representation of a transmembrane signal transduction system (Modified from Bell 1986).

Nishizuka 1986). Recently, the activated PKC was found to be involved during the cellular response to H-ras expression (Lacal et al 1987). Furthermore, the down regulation of PKC by phorbol esters (Pasti et al 1986) eliminates the cellular mitogenic response to ras p21 (Lacal et al 1987). The activation of PKC was implicated in the blockage of gap junctions and was recently shown to directly phosphorylate the junctional proteins of rat hepatocytes in a cell-free system (Takeda et al 1987). In addition to being strongly involved in transmembrane signaling systems, both the cellular and oncogenic ras p21 directly induce membrane changes. These changes are both rapid and long lasting with the oncogenic p21 (Bar-Sagi and Feramisco 1986) and they occur following its microinjection into quiescent, contact inhibited cells. The membrane alterations include membrane ruffling and pinocytosis (Bar-Sagi and Feramisco 1986). Membrane ruffling distorts the cell morphology and its normal boundary and surfaces of contact while pinocytosis affects intracellular homeostasis, particularly by increasing Ca^{++} influx. Calcium ions are known to activate the calcium sensitive PKC (May et al 1985), an effect associated with the blockage of junctional coupling (Rose and Loewenstein 1975, Rose et al 1977). The cell surface ruffling and pinocytosis were also found to be induced by exposing cells maintained in serum-free medium to either growth factors e.g., EGF (Haiglar et al 1979) or PDGF (Davies and Ross 1978), or serum (Brink et al 1976), NGF (Connolly et al 1979) or insulin (Goshima et al 1984). The fact that the ras protein directly induces similar changes might indicate its ability to bypass the cellular need for external mitogenic stimuli in order for the cell to proliferate.

Another characteristic of the ras gene product is that it

initiates DNA synthesis and cell proliferation (Feramisco et al 1984) and confers tumorigenic properties on preneoplastic or immortalized cell lines or cells expressing certain nuclear oncogene products e.g. myc, p53, E1a, or the large T antigen of SV40 virus (Land et al 1983a, 1983b, 1986, Parada et al 1984, Van Roy et al 1986, Beer et al 1986, Yancopoulous et al 1985, Ruley 1983, Segana and Yamaguchi 1987, Connan et al 1985). Interestingly, this latter property is shared by the phorbol ester, TPA, which induced transformed foci in cells immortalized by the myc oncogene (Connan et al 1985). An additional analogy was reported between ras and TPA when they both acted synergistically during in vitro transformation, thus suggesting a common cellular effect of both of them (Dotto et al 1985). Although the ras expression has been correlated with cellular transformation and proliferation, it was found to promote differentiation in certain cell systems (Noda et al 1985, Bar-Sagi and Feramisco 1985, Hagag et al 1986), an effect similar to that induced by TPA in some cells (Yamasaki 1984b). This differential effect could depend partially, or entirely, on the biochemical or molecular background of the host cell. In other words, the ras gene regulation, in certain host cells, may be altered by suppressor genes or "anti-oncogenes" or its product may not undergo certain post-translational modifications necessary for its activity.

The aforementioned properties of the ras oncogene product by themselves constitute strong basis to suspect a potential role the p21 might play during the stages of tumor promotion, i.e. the induction of proliferation and the inhibition of gap junctional communication. This role would be similar in some respects to that played by exogenous tumor promoters with the difference being that ras p21 is

constitutively expressed in the cell. If this particular cell were initiated, or to be initiated, then, most of the requirements for cellular transformation and proliferation could be met and clonal expansion would follow. As mentioned earlier, the rapid proliferation of initiated "immortalized" cells increases their chances for additional genetic damage and thus contributes to tumor progression, and metastasis, as well as heterogeneity. The ras oncogene was found highly expressed in several metastatic tumor cells and less expressed in malignant tumors before invasion and metastasis (Bondy et al 1985, Bradley et al 1986, Tahara et al 1986, Egan 1987, Collard et al 1987). This fact might indicate that the ras oncogene, in some systems, might play a more important role during tumor progression and metastasis rather than an earlier one in the initiation phase.

Reports to the contrary argued that the ras oncogene may influence the initiation stages of carcinogenesis (Balmain et al 1984, Brown et al 1986). In the reported experiments, the mouse skin keratinocytes were exposed in vitro to 7,12-dimethylbenz[a]anthracene (DMBA), a chemical known to reproducibly activate the H-ras oncogene, or were subjected in vivo to Harvey murine sarcoma virus containing an activated ras. These cells acquired tumorigenic properties within few weeks following exposure to the potent tumor promoter TPA. However, the in vitro and in vivo assays utilized for those experiments failed to demonstrate whether the tumorigenic cells expressing the ras oncogene were not immortalized or initiated before hand. It thus appears that the possible role of the ras oncogene in mitogenesis and "tumor promotion" is induced by membrane-dependent reactions that are transduced intracellularly, processed at various levels and lead to the observed

biological responses. The major consequences are, therefore, the loss of contact inhibition, alteration or loss of differentiation, blockage of gap junctional communication, stimulation of DNA synthesis and cell proliferation.

Despite the positive correlation between the expression of the ras oncogene and the inhibition of gap junction communication that is reported here, a direct cause/effect argument can not be made at this stage. Some of the cellular changes secondary to transfection with c-Ha-ras-1 could circumstantially interfere with cellular coupling. Among these, the alteration of membrane pinocytosis may have an important effect on cellular homeostasis by allowing a perturbation in the concentration of ions, e.g. Ca^{++}, or other factors that may block gap junctions. Moreover, the transformed morphology, i.e. membrane ruffling and anchorage independence, can act as mechanical factors limiting intercellular contact. In addition, auxillary cellular changes accompanying the transfection with, and the expression of, the ras oncogene, e.g. increase of pH (Hagag et al 1987), alteration of the nucleotide pool or gene expression, could act, individually or in combination, to inhibit gap junction coupling as well. Furthermore, the mitogenic effect of ras p21 that results in cell division can, by itself, lead to an inevitable uncoupling in the early stages of mitosis.

Further investigations are required to determine the role of the ras oncogene in tumorigenesis in general and in the modulation of gap junctions in specific. Oncogenes linked to inducible promoters, e.g. that of the murine mammary tumor virus (MMTV) or the metalthionine promoters, inducible by dexamethazone or zinc respectively, might be useful in the elucidation of a more direct correlation between the

expression of the H-ras p21 protein and the inhibition of gap junctions. Cells transformed by ras, with constitutive or inducible expression, can be injected in nude mice, or noncompromised animals, to test for correlation between the p21 expression, inhibition of gap junction and their tumorigenic capability in vivo. Also, antisense mRNA to ras or anti-p21 antibodies could be introduced into cells transformed with ras and these cells evaluated for their communication competence. The mechanisms of phenotypic transformation, membrane ruffling, pinocytosis or even blockage of gap junctions by the ras oncogenes have not yet been elucidated. It is therefore necessary to study the various cellular factors suspected to play a role in mediating the ras effect. Prolonged down-regulation of PKC e.g. by phorbol ester treatment, before and after transfection with ras oncogene, might directly verify the role of PKC in the process of transformation by ras. In addition, microinjection of ras p21 in the presence of protein synthesis inhibitors, e.g. cyclohexamide, and testing for modulation of gap junction conductance might help explore whether the production of newly synthesized proteins is required. Also, the role of intracellular Ca^{++} in ras-induced effects can be studied using TMB-8, a compound known to inhibit mobilization of calcium from intracellular stores (Chiou and Malagodi 1975). Calcium is one of the factors needed to activate PKC (May et al 1985).

Another intriguing aspect for research would be to characterize the intermediate biochemical pathways that intervene between the activated oncogenic p21 and the blockage of gap junctions. This characterization might lead to the identification of compounds that may potentially reverse the inhibition of gap junctions and induce cell

differentiation, e.g. cAMP (Saez et al 1986, Veld et al 1985, Johnson et al 1985, Wiener and Loewenstein 1983) and retinoic acid (Bollag 1972, Schiff and Moore 1985). Efforts need also be directed to elucidate the physiologic function of the normal ras proto-oncogene and its role in cell cycle, membrane function and cell proliferation. Progress in any of those aspects outlined here might prove valuable and could open up further possibilities for an in depth characterization of the role of gap junctions, tumor promoters and oncogenes in carcinogenesis.

CONCLUSIONS

The results described in the reported studies exhibit significant correlation between the expression of the H-ras product p21 and the inhibition of gap junctional communication. These data are in agreement with the proposed hypothesis, i.e. that ras may mimick the effects of certain tumor promoters by blocking the direct intercellular communication during carcinogenesis. This hypothesis thus integrates gap junction function, tumor promotion and oncogene expression during the process of malignant transformation. The results are not in contradiction with the known biochemical characteristics of the ras oncogene product. P21 has been reported to induce a constitutive activation of PKC through the elevation of intracellular DAG levels. This PKC activation also occurs during cell-exposure to phorbol ester tumor promoters and in cells expressing the src oncogene product. PKC activation leads to the phosphorylation of gap junction proteins in some cell systems and positively correlates with the inhibition of gap junctional communication.

The intercellular coupling mechanism appears to have a fundamental role in maintaining cellular homeostasis, control of differentiation, proliferation and contact inhibition. The interference with this junctional permeability was found to be associated with cellular exposure to mitogenic agents and tumor promoters. In addition, inhibited junctional communication correlates with certain malignant phenotypes and metastasizing cells and with those expressing oncogenic products. The

data presented here have elucidated an important potential function of the ras oncogene, i.e. the interference with gap junction conductance presumably acting through the membrane signal transduction mechanism. These findings, together with previous observations linking the inhibition of gap junctions to tumor promotion, implicate a role of the ras oncogene as a participant in this stage of tumorigenesis.

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