The University of Manitoba

Preliminary Evidence For The Possible Existence Of ras Oncogene-Homologous Sequence(s) In **Achlya** Genomic DNA

by

KUO KOEWANG TJONG

A Thesis

Submitted To The Faculty Of Graduate Studies
In Partial Fulfillment Of The Requirements
For The Degree Of Master Of Science.

Department of Microbiology

WINNIPEG, MANITOBA

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To God be the glory.

To my father and mother, Mr. Tjong, Djoen Fong and Mrs. Tjong(Loo), Giok Ing. To my aunts and uncles especially Mr. Tjong, Jin Fong, Miss Tjong, Kwee Joen, and Mr.& Mrs. Pranoto. Also to my brothers and sisters.

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ABBREVIATIONS

bp	basepair
cpm	counts per minute
dATP	deoxyadenosine 5´ triphosphate
dCTP	deoxycytosine 5´ triphosphate
dGTP	deoxyguanosine 5´ triphosphate
dTTP	deoxythymidine 5´ triphosphate
DNase	deoxyribonuclease
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetic acid
EtBr	ethidium bromide
kb	kilobasepair
M.W.	molecular weight
O.D.	optical density
RNase	ribonuclease
rpm	rotations per minute
sarkosyl	N-lauroylsarcosine
SDS	sodium dodecyl sulfate
TCA	trichloroacetic acid
Tris	tris (hydroxymethyl) amino methane

ABSTRACT

Southern blot analysis of **Achlya klebsiana** genomic DNA by non-stringent hybridization conditions using mammalian Ha-ras and viral Ha-ras oncogenes as probes detected hybridization bands. This provides a preliminary evidence of ras-ralated sequence(s) in **Achlya klebsiana** genome. The hybridization bands detected, however, are relatively weak, this may indicate a low degree of homology between ras-related sequence(s) in **Achlya** and mammalian Ha-ras or viral Ha-ras oncogenes or both.

INTRODUCTION

Oncogenes are genes which are involved in the development of a cancerous cell. This group of genes, which were initially described as endogenous constituents of retroviral genome, have proved to be normal members of cellular genomes. For every viral version of oncogenes isolated, there exists a cellular counterpart(s) in vertebrate or invertebrate animals or both (Bishop,1983).

The expressions of oncogenes have been described in normal cells of all species examined (Bishop,1983). These findings indicate that oncogenes are vital members of cellular genome. In addition, the distribution of oncogenes in species, which according to the evolutionary theory represent a broad expanse of time, supports the idea that oncogenes existed early in evolution. This idea prompted a search for similar genes in lower animals and eukaryotic microbes.

In 1981, genes homologous to the ras oncogene were discovered in **Drosophila melanogaster** (Shilo and Weinberg,1981). Two years later, a similar discovery was made in yeast **Saccharomyces cerevisiae** (DeFeo-Jones et al.,1983), and more recently in the slime mould **Dictyostelium discoideum** (Pawson et al.,1985). The biochemical properties

and function(s) of ras homologues in these eukaryotic microbes show much similarity with ras in mammalian species (Powers et al.,1984, Papageorge et al.,1984, Pawson et al.,1985). In addition, ras homologues in yeast have been demonstrated to play an as yet undeciphered role in spore viability (Tatchell et al.,1984). Strong genetic manipulation, which can be applied in yeast, offers scientists a good alternative model for studying ras oncogene.

In light of such discoveries, this study was designed to probe whether ras-homologous gene(s) existed in a water mould **Achlya klebsiana.**

Achlya klebsiana is a species of water mould generally found in fresh water, soil and mud. The life cycle of **Achlya klebsiana** has been well documented. Its physiology and biochemistry have been investigated in terms of its enzyme regulation, membrane transport, influence of various agents on its life cycle and development, and so forth (LéJohn,1971, Cameron and LéJohn,1972, LéJohn et al.,1978, LéJohn and Braithwaite,1984). Thus, it is of great benefit if function(s) of ras-encoded protein(s), and the relationship between sporulation process and ras homologue(s) can be studied in the water mould **Achlya**, whose life cycle is well defined physiologically as well as biochemically and is relatively easily manipulated.

H I S T O R I C A L

The concept that certain viruses cause cancer is not new. The term "oncogenic virus" was introduced in 1972 by Eckhart. The idea behind it, however, originated at the turn of the twentieth century when Rous (1911) discovered that cell and bacteria-free filtrates obtained from chicken sarcoma cells could induce new sarcomas in healthy chickens.

Oncogenic viruses include DNA viruses and RNA viruses. RNA viruses, however, take precedence over DNA viruses in their link to cancer for at least two reasons. First, the RNA tumor viruses cause naturally occurring leukemias and sarcomas in birds, rodents and cats. Second, large numbers of RNA tumor viruses are known to cause cancer (Eckhart,1972).

A study by Varmus et al.(1972) noted that genetic material homologous to RNA tumor viruses could be detected in many normal cells which did not contain detectable viral components or mature virus. The wide-spread occurrence of the genetic material found in the RNA tumor viruses led Hueber and Todaro (1969) to postulate the "oncogenic hypothesis" which proposed that the retroviral oncogenes resided in the germ lines of all species. This proposal proved to be somewhat inaccurate. Vertebrates do harbor DNA sequences homologous to retrovirus oncogenes, but these are normal cellular genes rather than retroviral genes. The use of recombinant DNA techniques has demonstrated that most

vertebrate oncogenes are unique loci situated at constant chromosomal positions within any given species; some display minor structural polymorphisms recognized only in cellular genes such as the regions where transcription starts and stops; several cellular oncogenes have introns within the coding sequence of the gene; and none has been found within or linked to the proviruses of endogenous retroviruses (Bishop,1981).

There is little doubt, if any, that retrovirus oncogenes and cellular genes are related. But where did the oncogenes originate? The first clue, as to the origin of the oncogenes, was provided when sequences closely related to the Rous sarcoma virus oncogene were discovered in the avian (Stehelin et al.,1976) and mammalian (Spector et al.,1978c) species. According to evolutionary theory, these two species represent a broad expanse of time. Second, viral oncogenes are generally restricted to single strains of retroviruses isolated from particular species. Third, retrovirus oncogene possesses the greatest homology to the cellular gene of the species from which the retrovirus is allegedly isolated (Bishop,1981). These findings strongly suggest that retrovirus oncogenes were derived from cellular oncogenes.

More than 20 viral genes responsible for cell neoplastic transformation have been isolated from viruses which are known to transform mammalian cells upon infection. These

genes are termed viral-oncogenes (v-oncs) while their cellular counterparts are called cellular-oncogenes (c-oncs) (Bishop,1983). The recent listing of oncogenes is shown in table 1.

Transcription of several cellular oncogenes has been detected in normal cells. In some cases, normal cell proteins which are closely related to proteins encoded by the homologous retroviral oncogenes, have been identified (Bishop,1981). These findings show that cellular oncogenes are household genes vital for normal functioning of cells.

The evolutionary theory requires the preservation of vital genes. This phenomenon seems to appear in oncogene preservation. For example, the widely studied ras oncogene has been discovered from organisms as disparate as yeast (DeFeo-Jones et al.,1983) and humans (Pulciani et al.,1982); myc oncogene is found in **Drosophila** (Shilo and Weinberg,1981) and humans (Dalla-Favera et al.,1982); and src oncogene is expressed in chickens and humans (Parker et al.,1981).

Table of oncogenes, retroviruses associated with, tumors in human and other vertebrates associated with the oncogenes, and oncogenic proteins encoded by them. Adopted from "The proteins of oncogenes" by Hunter, 1984.

NAME OF ONCOGENE	RETROVIRUS	TUMOR	ONCOGENIC PROTEIN		
			CELLULAR LOCATION	FUNCTION	CLASS
src	CHICKEN SARCOMA	—	PLASMA MEMBRANE	TYROSINE-SPECIFIC PROTEIN KINASE	CLASS 1 (CYTOPLASMIC TYROSINE PROTEIN KINASES)
yes	CHICKEN SARCOMA	—	PLASMA MEMBRANE (?)		
fgr	CAT SARCOMA	—	(?)		
abl	MOUSE LEUKEMIA	HUMAN LEUKEMIA	PLASMA MEMBRANE		
fps	CHICKEN SARCOMA	—	CYTOPLASM (PLASMA MEMBRANE?)		
fes	CAT SARCOMA	—	CYTOPLASM (CYTOSKELETON?)		
ros	CHICKEN SARCOMA	—	?		
erb-B	CHICKEN LEUKEMIA	—	PLASMA AND CYTOPLASMIC MEMBRANES	EGF RECEPTOR'S CYTOPLASMIC TYROSINE-SPECIFIC PROTEIN-KINASE DOMAIN	CLASS 1-RELATED (POTENTIAL PROTEIN KINASES)
fms	CAT SARCOMA	—	PLASMA AND CYTOPLASMIC MEMBRANES	CYTOPLASMIC DOMAIN OF A GROWTH-FACTOR RECEPTOR (?)	
mil	CHICKEN CARCINOMA	—	CYTOPLASM	(?)	
raf	MOUSE SARCOMA	—	CYTOPLASM	(?)	
mos	MOUSE SARCOMA	MOUSE LEUKEMIA	CYTOPLASM	(?)	
sis	MONKEY SARCOMA	—	SECRETED	PDGF-LIKE GROWTH FACTOR	CLASS 2 (GROWTH FACTORS)
Ha-ras	RAT SARCOMA	HUMAN CARCINOMA, RAT CARCINOMA	PLASMA MEMBRANE	GTP-BINDING	CLASS 3 (CYTOPLASMIC, GTP-BINDING)
Ki-ras	RAT SARCOMA	HUMAN CARCINOMA, LEUKEMIA AND SARCOMA	PLASMA MEMBRANE		
N-ras	—	HUMAN LEUKEMIA AND CARCINOMA	PLASMA MEMBRANE		
fos	MOUSE SARCOMA	—	NUCLEUS	(?)	CLASS 4 (NUCLEAR)
myc	CHICKEN LEUKEMIA	HUMAN LYMPHOMA	NUCLEUS	DNA-BINDING	
myb	CHICKEN LEUKEMIA	HUMAN LEUKEMIA	NUCLEUS	(?)	
B-lym	—	CHICKEN LYMPHOMA, HUMAN LYMPHOMA	NUCLEUS (?)	(?)	
ski	CHICKEN SARCOMA	—	NUCLEUS (?)	(?)	
rel	TURKEY LEUKEMIA	—	(?)	(?)	UNCLASSIFIED
erb-A	CHICKEN LEUKEMIA	—	(?)	(?)	
ets	CHICKEN LEUKEMIA	—	(?)	(?)	

Table 1

Structural characteristic and expression of cellular oncogenes

Since the discovery of cellular homologues of viral oncogenes, the emphasis on the study of oncogenes has shifted from viral oncogenes to their cellular counterparts. Through the use of recombinant DNA technology virtually all of the known cellular oncogenes have been molecularly cloned from at least one species (Bishop,1983). These cellular prototypes of oncogenes have been compared with viral counterparts by heteroduplex analysis, restriction endonuclease mapping and complete sequence analysis (Oskarsson et al.,1980). Although none of the cellular oncogenes cloned has been described fully, cellular oncogenes do share several common features typical of a cellular gene; they segregate in a predictable fashion as classical Mendelian loci and possess introns as well as transcription start and stop signals.

Cellular oncogenes possess topographical diversity equivalent to that of other cellular genes. Their sizes range from a few kilobasepairs to over forty kilobasepairs (Goff et al., 1980, Vennstrom and Bishop, 1982, Chen et al.,1983). Some cellular oncogenes have no introns (Jones et al., 1980, Oskarsson, 1980, and van Beveren et al.,1982) while others have more than a dozen. The size of individual introns also may vary from tens of base pairs to thousands of basepairs (Goff et al.,1980, Vennstrom and Bishop,1982, Chen

et al.,1983).

Transcripts of virtually all but one (i.e. mos) cellular oncogenes have been detected in normal or tumor cells or both (Spector et al., 1978a, Spector et al., 1978b, Chen, 1980, Gonda et al., 1982, Muller et al., 1982, Shibuya et al., 1982, Ellis et al., 1982, Westin et al., 1982a, Westin et al., 1982b, and Eva et al., 1982). The transcription of cellular oncogenes appears to follow several general principles. (1) The transcription of cellular oncogenes is non-tissue specific. It occurs in a variety of tissues and in every species of vertebrate examined. (2) The amounts of transcript produced are significantly small (Bishop, 1983). (3) Cellular oncogenes are not expressed in a coordinate fashion but each gene may function only in certain tissues (Weiss et al., 1982). (4) Some cellular oncogenes are shown to be expressed in specific stages of cell cycle and development (Reymond et al., 1984, Tatchell et al., 1984).

Mechanisms of activation of cellular oncogenes

It is known that cellular oncogenes are normal constituents of cellular genome and have normal and vital roles in cell cycle and development. They also possess, however, the hazardous potential of turning malignant. Although little about their normal function(s) has been discovered, knowledge on how cellular oncogenes turn

malignant will help our understanding of their normal function(s).

Based on current information, four mechanisms of cellular oncogenes activation have been suggested. The first mechanism is point mutation. An example of point mutation is the activation of c-ras oncogene in carcinoma of the human bladder. A single mutation within the coding domain of the gene, a transversion of G to T changing a glycine residue to valine in the deduced amino acid sequence, is responsible for the carcinoma (Tabin et al., 1982, Reddy et al., 1982).

The second mechanism is chromosomal rearrangement. This mechanism relates to the activation of a c-onc, the myc, whose active version of oncogene has been found in mouse myelomas and human Burkitt´s lymphoma (Leder et al., 1983. The cellular oncogene becomes separated from its usual position at the end of one chromosome and is transposed to the end of a second chromosome. There it becomes juxtaposed with genes responsible for the synthesis of immunoglobulin. In the course of an immune response these genes are transcribed at a high rate (Leder et al., 1983).

The third mechanism of activation is gene amplification. Instead of the normal two copies, a cellular oncogene has been found to be present in large numbers of copies per cell (Alitalo et al., 1983). This amplification of the gene and the resulting high level of gene expression appears also to

have an oncogenic effect.

The fourth mechanism of activation involves retroviruses. Retrovirus, in the course of infection, may pick up a cellular sequence which includes oncogene and integrate it into its own genome. Consequently, during the next course of infection, the introduction of oncogene, which may have been activated by association with a regulatory region of the viral genome or by undergoing mutation while being carried by the retrovirus, can cause cancer in many animals (Weinberg, 1983). Alternatively, the retrovirus infecting a cell may transfer its powerful regulatory region juxtaposed to cellular oncogene and promote its expression in great abundance (Marx, 1984a).

Functions of cellular oncogenes

Evolutionary theory states that it is against the law of natural selection for a group of genes to be preserved in response to their oncogenic potential. Since oncogenes exist, in accordance to the law of natural selection, one may conclude that cellular oncogenes possess function(s) vital to the growth and development of the species in which they reside.

In order to study the function of a gene one needs to study the function of the protein(s) encoded by it. It has not been easy to discern the biochemical functions of

cellular oncogenes. One reason may be that proteins coded by oncogenes are present in such a minute amount (Bishop, 1983). Recent development in recombinant DNA techniques, however, has made oncogenic proteins available in large quantity.

Based on the accumulated information, the functions of oncogenic proteins can be divided into four categories. (1) Oncogenic proteins with tyrosine-specific protein kinase activity. These include the products of oncogenes src, yes, fgr, abl, fps, fes, and ros. This group of proteins display enzymatic activity catalyzing the addition of a phosphate molecule to tyrosine residues in a protein (Hunter, 1984). The most recent member of this group is erb-B (Gilmore et al., 1985). (2) Oncogenic protein with function like platelet-derived growth factor. The sole member of this group is the protein encoded by sis oncogene. It appears that c-sis is none other than the gene for platelet-derived growth factor itself (Hunter, 1984). (3) The third group consists of GTP-binding proteins. The proteins produced by the family of ras genes fit into this category. They bind GTP tightly forming a stable complex (Shih et al., 1980 andScolnick et al., 1981) and share homologous function with G protein. G proteins are known to bind GTP and together with GTP interacts with the effector adenylate cyclase (Hurley et al., 1984). (4) Included in the fourth group are proteins of fos, myc, myb, B-lym and ski oncogenes. They are

known to be located in the nucleus. The protein encoded by myc oncogene binds DNA in the nucleus (Hunter, 1984).

The proteins of the remaining oncogenes do not fit into any of the above four categories. The proteins of oncogenes fms, mil, raf and mos may have potential of being protein kinases, while little has been known of the protein products of rel, erb-A and ets (Hunter, 1984).

Oncogenes in vertebrates and non-vertebrates

In the past five years, an investigation has begun to uncover oncogenes in human and animal tumor cells rather than in retroviruses. Genes in the DNA of various kinds of tumor cells were found that, on being introduced by "transfection" into normal cells, transformed them into cancer cells (Cooper, 1982, Pulciani et al., 1982). These tumor cells that yielded oncogenes were not induced by retroviruses, therefore there was no reason to expect the tumor oncogenes to be the same as the retrovirus ones. Nevertheless, there was some overlap between the two groups (Hunter,1984).

The most frequently isolated tumor oncogene has proved to be a homologue of the retroviral oncogene ras which is carried by the Harvey strain of murine sarcoma virus. The ras gene has been detected in a wide variety of vertebrate species (Weiss et al.,1982), including **Xenopus laevis**

(DeFeo-Jones et al.,1983), and in non-vertebrates such as **Drosophila melanogaster** (Shilo and Weinberg,1981), **Saccharomyces cerevisiae** (DeFeo-Jones et al.,1983), and recently in **Dictyostelium discoideum** (Pawson et al.,1985).

Viral-ras and cellular-ras oncogenes

A number of transforming viruses of rodents, including the Harvey and Kirsten strains of murine sarcoma virus (Ha-MSV and Ki-MSV) and several apparently identical isolates of Rasheed rat sarcoma virus, are related to a viral oncogene termed ras. All of these viruses are replication-defective, transform cultured fibroblasts, and produce sarcoma in rodents (Weiss et al.,1982).

Ha-MSV and Ki-MSV were generated during propagation of murine leukemia viruses (MLVs) in rats. The first MSV to be found was isolated by Jennifer Harvey (1964). After passaging the Moloney strain of MLV in rats, Harvey obtained a virus preparation that produced pleomorphic sarcomas as well as leukemias. Subsequently, Kirsten and Mayer (1967) isolated MSV in stocks of the Kirsten MLV strain that had been passaged in rats. It should be noted that the Ha-MSV´s and the Ki-MSV´s genomes are composed of three parental components. The terminal sequences were provided by the MLV genomes; about 1 kb. of ras sequence was isolated in the 5´ half of the RNA, and sequences from the replication-defective

endogenous virus-like genome of rat flanked both sides of ras (Ellis et al.,1980).

The oncogenes of retroviruses are genetic luxuries. In no known instances is the function of an oncogene required for virus replication. It is now clear that retroviral oncogenes originated from normal genes of vertebrate cells (c-onc), and that oncogenes and their vertebrate progenitors remain closely related if not identical (Weiss et al.,1982).

The search for oncogenes in cellular DNA began with the use of molecular hybridization. An initial search, using labelled copy DNA of v-src, provided evidence for the existence of DNA and RNA sequences related to the v-src gene in each family of vertebrate examined; including fish, birds and mammals (Stehelin et al.,1976, and Spector et al.,1978c). Based on evolutionary theory, the mere fact that homologous DNA could be detected across such large phylogenetic distances indicates that the genetic locus in question is highly conserved (Weiss et al.,1982).

Hughes et al.(1979) and Weiss et al.(1982) discovered that cellular oncogenes behave as classical Mendelian loci. That is to say, cellular oncogenes occupy constant positions within the genomes of particular species (Hughes et al.,1979) and they segregate in the predictable fashion (Weiss et al.,1982).

As one would expect, cellular homologues of oncogenes

differ from viral oncogenes in features which typify eukaryotic genes. Heteroduplex analysis and restriction mapping have demonstrated that the homology between several viral oncogenes and their cellular oncogenes is interrupted by one or more intervening sequences in the cellular locus (Goff et al.,1980, DeFeo et al.,1981, Franchini et al.,1981, Parker et al.,1981, Shalloway et al.,1981, and Takeya et al.,1981). Many retroviral oncogenes have been formed by the fusing of a portion of a replicative gene (typically, gag) to nucleotide sequences of a c-oncogene. In particular, sequences in the 5′ domain of the c-oncogene may be missing from the viral oncogene.

The family of c-ras genes has two arms: one transduced as v-ras in the Harvey strain of murine sarcoma virus and the other as v-ras in the Kirsten murine sarcoma virus (Ellis et al.,1981). Harvey and Kirsten c-ras are distributed in three patterns among different species; as unique loci (possible examples include chicken and mice) (Ellis et al.,1981, Chattopadhyay et al.,1982) and as duplication within the Harvey and Kirsten lineages (in rats and human) (DeFeo et al.,1981, Ellis et al.,1981, Chang et al.,1982b) and as substantially amplified gene families (in the rodent _Mus pohari_ and the chinese hamster) (Chattopadhyay et al.,1982). The third branch of c-ras in the family isolated as the transforming gene of a human neuroblastoma cell line

(SK-N-SH) which is related to (but distinct from) the homologues of the v-Ha and v-Ki-ras genes is called N-ras (Shimizu et al.,1983). Another ras-related gene family has also been reported recently by Madaule and Axel (1985). The so-called rho genes were isolated from DNA of abdominal ganglia of **Aplysia.** In addition, homologues to the rho gene family have been detected in yeast, **Drosophila** and human cells.

(i) Activated c-ras in variety of tumor cells

It is interesting that a wide variety of tumor cells contain activated ras gene. In human, for instance, carcinoma of the bladder contains activated c-Ha-ras (Santos et al.,1982), a carcinoma of the lung and colon contain activated c-Ki-ras (Der et al.,1982), while neuroblastoma cells contain activated N-ras (Shimizu et al.,1983). Transcripts hybridization to v-ras probe were detected in human sarcoma, carcinoma, melanoma and astrocytoma cell lines (Eva et al.,1982).

In their native form, cellular oncogenes appear to be incapable of transforming cells (Cooper,1982, Weinberg,1982). Activation of ras,proved by subsequent ability to transform NIH/3T3 cells, is produced by a point mutation in the ras gene (Santos et al.,1982, Santos et al.,1984, Reddy et al.,1982) or by enhanced expression of the normal ras gene

(Chang et al.,1982a, DeFeo et al.,1981, McCoy et al.,1983). The latter mechanism of activation, however, contradicts observation that in many different human tumor cell lines, the expression of c-ras is similar, independent of whether the cell line contains an activated ras detectable by transfection (Der and Cooper,1983).

In human neoplasm, activation of ras genes through structural mutations appear to be the common mechanism of oncogenic induction. The mutations appear to occur primarily at codon 12 or 61, with different amino acid substitutions resulting in ras gene activation in different tumors (Finkel et al.,1984). Introduction of antibodies specific for amino acid 12 of ras protein is able to transiently reverse ras oncogene-induced cell transformation (Feramisco et al.,1983).

(ii) Proteins encoded by ras oncogenes

Virtually all oncogenes are transcribed and translated in normal or tumor cells or both. The ras gene family encode very similar proteins with molecular weights about 21,000 daltons (p21 c-ras) (Langbeheim et al.,1980, Furth et al.,1982). These proteins are similar with their counterparts p21 v-ras. They share at least some antigenic determinants, produce related peptide maps and possess the ability to bind guanine nucleotides with high affinity (Shih et al.,1980). The main difference between proteins produced

by v-ras and c-ras is that p21 of c-Ha-ras lacks in autophosphorylation at position 59 of the protein. This is probably due to the lack of threonine at that position (McGrath et al.,1984). In addition to the ability to bind GTP specifically, a GTPase activity has been detected which extensively co-purifies with the p21 ras polypeptides (McGrath et al.,1984). While structural mutations which activate ras gene transforming activity do not affect the protein´s intrinsic ability to bind guanine nucleotide, GTPase activity is selectively impaired by the mutation (Finkel et al.,1984, and McGrath et al.,1984).

The v-Ha-ras gene of BALB-MSV has been translated in **E. coli** (Lacal et al.,1984). Injection of the purified protein itself is sufficient to induce a transformed morphology in NIH/3T3 cells. In addition, the injected protein stimulates quiescent cells to enter the S phase of the cell cycle. This result clearly demonstrates that the ras gene functions directly through the protein product (Stacey and Kung,1984). Microinjection of the mutated human c-ras protein into fibroblast cells also result in a rapid alteration in cell morphology, stimulation of DNA synthesis and cell division (Feramisco et al.,1984). A similar result is also produced by normal protein, but the altered protein is effective at a lower concentration (Stacey and Kung,1984).

The intracellular location of both normal and activated

p21 ras is at the inner face of the plasma membrane. The human ras proteins are produced in the soluble polysome fraction of cells and subsequently migrate to the cytoplasmic side of the plasma membrane. The proteins undergo post-translational modification which involves lipidation (Papageorge et al.,1982, Sefton et al.,1982). A comparison of proteins encoded by normal and activated ras genes reveal no changes in subcellular localization and post-translational modification (Finkel et al.,1984).

The location of p21 at the inner face of the plasma membrane led to the idea that the protein could play a major role in transducing signals from growth factors acting at the cell surface to the rest of the cell. This idea was supported by two facts: (1) The ras proteins bear some structural homology with the G protein which is known to regulate the hormone-sensitive adenylate cyclase activity (Gilman,1984). (2) Epidermal growth factor or insulin stimulate the GTP-dependent phosphorylation of the murine v-ras protein and the guanine nucleotides binding activity of the ras protein in ras transformed cells (Kamata and Feramisco,1984).

In yeast, there is evidence which indicates that the transforming protein produced by the ras gene is an activator of the enzyme adenylate cyclase. Mutant ras protein has been shown to continuously activate adenylate cyclase, whereas the

normal protein does not have this effect. The ras protein may activate adenylate cyclase as the G protein does. Although the normal ras protein apparently must itself be turned on in some fashion to achieve this, the transforming ras protein, perhaps, because it has lost the ability to split GTP, may be constantly "on" even when it should be "off" (Marx,1984b).

(iii) Genes homologous to mammalian ras genes in non-vertebrates

Ha-ras-homologous sequences in Drosophila melanogaster

Shilo and Weinberg in 1981 sought to find genes homologous to mammalian oncogenes in a lower eukaryote **Drosophila melanogaster.** Probes prepared from oncogene of v-Ha-ras detected three DNA fragments in **Drosophila** DNA (Shilo and Weinberg,1981).

Three **Drosophila** genes homologous to Ha-ras probe have been isolated and their locations mapped in the **Drosophila** genome; they are mapped at positions 85D, 64B, 62B on chromosome 3 (Neuman-Silberberg et al.,1984). Two of these genes, Dras1 and Dras2, code for a protein with a predicted molecular weight of 21,600 daltons.

Alignment of the amino acid sequence of Dras1 and Dras2 with the vertebrate Ha-ras protein shows that they have an overall homology of 75% and 50% respectively for Dras1 and

Dras2. The homology is greatest at the amino terminus and central portion of the proteins. This result implies that the N-terminus of the p21 protein forms a distinct regulatory or functional domain (Neuman-Silberberg et al.,1984).

All three ras sequences in **Drosophila** appear to be functional genes. Dras1 and Dras2 display an open reading frame, and both were shown to be constitutively expressed at different stages of development (Neuman-Silberberg,1984).

ras-homologous sequences in Saccharomyces cerevisiae

A v-Ha-ras-specific DNA probe was used in relaxed hybridization conditions to detect ras-related sequences by Southern blot analysis of yeast genomic DNA. Two ras-related DNA fragments were isolated and cloned. These two genes termed c-ras sc-1 and -2 share less than 75% homology to v-Ha-ras gene. c-ras sc-1 possesses a single open reading frame in the ras-related region. c-ras sc-2, on the other hand, may contain less ras information or may be more divergent from v-Ha-ras than c-ras sc-1 (DeFeo-Jones et al.,1983).

Amino acid sequences derived from nucleotide sequences of yeast ras-1 and ras-2 show that yeast ras-1 and -2 are more homologous to each other than they are to mammalian ras proteins. The yeast ras proteins share nearly 90% homology to the first 80 positions of the mammalian ras proteins, and

only 50% homology to the next 80 amino acids. At the position where yeast ras proteins start to drop in the degree of homology to mammalian ras protein they,too, start to diverge from each other (Powers et al.,1984).

A third gene, termed YP2, has also been described in **S. cerevisiae** (Gallwitz et al.,1983), which can encode a protein with significant but much weaker homology to the mammalian ras proteins. Comparing with the proteins of yeast ras genes the protein encoded by YP2 is also quite diverged from both yeast ras proteins (Powers et al.,1984).

It is suggested that these domains of divergence within ras proteins of the same species or interspecies correspond to functional domains of the ras protein as well. Powers et al. suggested that the N-terminal domain is the effector region of the ras proteins, involved in interactions of a catalytic or regulatory nature. It is in this region that certain amino acid substitutions can activate the transforming potential of the mammalian ras proteins. On the other hand, the C-terminal variable domain contains the determinants of physiological specificity. Through this region, the ras proteins may receive their normal physiological signals, which are then transduced or mediated to the N-terminus (Powers et al.,1984).

A further study shows that yeast ras-1 and -2 encode an essential function in yeast. The viability of yeast cells

containing disruption of either of these genes indicates that the function(s) specified by either is sufficient to support both mitotic and meiotic cell division cycles, but at least one wild type gene is required (Tatchell et al.,1984).

Yeast spores lacking functional endogenous ras gene will not germinate. Spores containing chimeric mammalian/yeast ras genes or even the mammalian Ha-ras gene under the control of galactose-inducible promotor will germinate. These results indicate that the biochemical function of ras proteins is essential for vagetative haploid yeast and that this function has, evolutionarily speaking, been preserved (Kataoka et al.,1985).

The yeast protein encoded by ras-2 shares antigenic sites with the mammalian Ha-ras (Powers et al.,1984, Papageorge et al.,1984) and, like the mammalian ras proteins, binds guanine nucleotides (Tamanoi,1984). A mutated yeast ras-2 gene, ras-2^{val-19}, encoding a protein with an amino acid substitution identical to one that activates Ha-ras, can dramatically alter the growth properties of haploid and diploid yeast cells (Kataoka et al.,1984, Toda et al.,1985). These results again further confirm that the yeast ras proteins are a good model for understanding the mammalian ras proteins (Kataoka et al.,1985).

The close similarity between ras proteins from yeast and from mammalian cells opens up a wide avenue of study and

gives a better understanding of mammalian ras proteins. Genetic manipulations which can be performed with yeast but not mammalian cells gives scientists a strong advantage in using yeast as a study model for mammalian ras.

ras-related protein in Dictyostelium discoideum

A monoclonal antibody that specifically recognize the p21 transforming protein of Ha-MSV has been developed (Furth et al.,1982). This antibody is capable of specifically immunoprecipitating a yeast protein encoded presumably by one of these yeast ras genes (Powers et al.,1984). In a recent work, using the monoclonal antibody against mammalian p21 ras, a protein with molecular weight of 23,000 daltons (p23) has been precipitated from lysate of **Dictyostelium discoideum** amoebae (Pawson et al.,1985).

Tryptic peptide analysis of the protein indicated that p23 was closely related in its primary structure to mammalian p21 ras. Many common features are shared between these two proteins. Like p21 ras, **D. discoideum**´s p23 is relatively minor cellular protein. Normal and transforming mammalian p21 ras proteins are synthesized as 22,000 M.W. primary gene products and undergo post-translational modification to a 21,000 M.W. species (Shih et al.,1982). Similarly, p23 is derived by post-translational modification of a 24,000 M.W. primary gene product of **D. discoideum** (Pawson et al.,1985).

There is a strong correlation between the expression of the ras-related protein p23 and cell proliferation of **D. discoideum** (Pawson et al.,1985).

The similarities in the size, structure, abundance, and apparent post-translational processing of **D. discoideum** p23 and mammalian p21 argue in favour of the concept that p23 is encoded by a **D. discoideum** ras gene and provide further evidence that ras-related sequences are widely distributed in eukaryotic cells (Pawson et al.,1985).

MATERIALS AND METHODS

(I) Organism

The organism used was a coenocytic filamentous fresh water mould **Achlya klebsiana** (LéJohn and Braithwaite,1984) originally supplied by Dr. J.S. Lovett of Purdue University, which has been maintained in this laboratory since 1969.

When grown in liquid medium, the growth pattern of **Achlya klebsiana** can be followed microscopically. The life cycle and growth conditions have been described (Cameron and LéJohn,1972).

(II) Plasmids

Three kinds of plasmid were used. They are:

(a) pbC-Nl

Plasmid pbC-Nl hosted in **Escherichia coli** strain C-600 was purchased from American Type Culture Collection. It contains human c-Ha-ras proto-oncogene (Pulciani et al.,1982) isolated from human fetal liver cells. The size of pbC-Nl is 10.7 kb., which is made up of 6.4 kb. human c-Ha-ras proto-oncogene inserted into Bam Hl site of 4.3 kb. plasmid pBR 322.

The host **E. coli** strain C-600 carrying pbC-Nl was selected by growing the culture in the presence of the antibiotic ampicillin.

(b) **pT24-C3**

Plasmid pT24-C3 hosted in **E. coli** was also purchased from American Type Culture Collection. pT24-C3 carries human bladder oncogene isolated from T24 bladder carcinoma cell line (Santos et al.,1982 and Reddy,1983). The total size of 10.9 kb. is made up of 6.6 kb. human bladder oncogene inserted into Bam H1 site of 4.3 kb. plasmid pBR 322.

Selection for the host **E. coli** carrying this plasmid was carried out by growing the culture in the presence of the antibiotic ampicillin.

(c) **pHB-11**

The Harvey strain of murine sarcoma virus (Ha-MuSV) is a replicative-defective retrovirus that was isolated from mouse tumor induced by murine leukemia virus which had been passaged through rats (Harvey,1964). Ha-MuSV genomic DNA has been molecularly cloned (Stehelin et al.,1976). The nucleic acid sequences of Ha-MuSV is derived from both murine leukemia virus and rat cellular DNA (Shih et al.,1978, and Chien et al.,1979). The clone HB-11 is a portion of Ha-MuSV genomic DNA which contains DNA sequences representing common rat 30S RNA sequences and DNA sequences responsible for trasnforming activity (Ellis et al.,1981).

Plasmid pHB-11 hosted in **E. coli** strain HB 101 was obtained from American Type Culture Collection. It contains

Bam H1-Eco R1 fragment of Ha-MuSV genome inserted into vector plasmid pBR 322 (from which Bam H1-Eco R1 region had been removed) (Ellis et al.,1981). The total size of pHB-11 is 6.2 kb.. It is made up of 4.0 kb. pBR 322 and 2.2 kb. insert.

Selection for the **E. coli** carrying this plasmid was carried out by growing the culture in the presence of the antibiotic ampicillin.

(III) Media for growth

(a) G_2Y medium

This medium consisted of 5.0 g glucose and 0.5 g yeast extract per liter of distilled water sterilized by autoclaving. Sterile $CaCl_2$ (50 µM final concentration) and sterile $MgCl_2$ (50 µM final concentration) were added prior to use.

(b) Defined medium

This medium, defined by Barksdale (1963), was made up of: 0.5 g sodium glutamate (mono), 2.8 g glucose, 1 ml (of 20 mg/ml in 0.1 N HCl solution) L-cystine, 1.5 mM KH_2PO_4, 2.0 mM KCl, 0.5 mM $MgSO_4$, 0.5 mM $CaCl_2$, 10 mg Na_2EDTA, 1.2 g Tris-base, and 10 ml metal mix, in one liter distilled water, pH 6.8.

Metal mix consisted of 100 ml of freshly prepared solution containing 100 mg sulfosalicyclic acid and 200 mg of a ground mixture of $Fe(NH_4)_2(SO_4)_2.6.H_2O$ (28.9 g), $ZnSO_4.7.H_2O$ (8.8 g) and $MnSO_4.H_2O$ (3.1 g).

Sterile glutamine (0.5 mM final concentration) and glucose, which was autoclaved separately, were added prior to use.

(c) Luria broth

One liter of this medium contained 10 g Bacto tryptone, 5.0 g Bacto yeast extract, 5.0 g NaCl. The medium was adjusted to pH 7.0 before autoclaving.

Achlya klebsiana was grown as stock culture in G_2Y medium and in Defined medium to culture cells required for profuse spore production.

Luria broth medium was used to grow **E. coli.**

(IV) Preparation of culture

(1) Achlya klebsiana

(a) Stock cultures

Cultures were maintained in petri plates containing 20 ml of G_2Y medium. The plates were kept at room temperature in the laboratory. Thick mats of **Achlya** developed in about

2-3 days.

Aseptic transfer to fresh medium was done approximately bi-weekly. Spores were loosened from the hyphal mat by agitation in sterile plastic centrifuge bottles; five mats in 100 ml of sterile water. After agitation, mats were removed and 2 ml of spore suspension was inoculated into each petri plate.

(b) Preparation of culture for study

Spores produced by **Achlya** can be well maintained in the spent medium of Defined medium. In order to obtain a rich spore harvest, Defined medium was inoculated with spores from G_2Y cultures and grown for at least 3 days at room temperature. The undisturbed spores can remain viable for several weeks.

(c) Large-scale germling preparation

Large-scale germling production was necessary for DNA isolation. Spores from 20 petri plate culture of **Achlya** grown in Defined medium were loosened by agitation in sterile plastic centrifuge bottles and filtered through 61 µm or 80 µm nylon filters to separate spores from mycelia. The spore filtrate was inoculated into one liter G_2Y medium, and the medium was incubated for 3 to 4 hours at $28^{o}C$ with constant shaking. If necessary, the period of incubation was extended.

(2) Escherichia coli

(a) Stock culture

Stock culture of plasmid-carrying **E. coli** was maintained in small vials containing sterilized glycerol and stored at $-20^{\circ}C$.

(b) Large-scale culture

Large-scale culture of **E. coli,** prepared for the purpose of plasmid DNA isolation, was grown as follows:

First, small volume of **E. coli** culture was prepared by inoculating 10 ml of Luria broth (LB) medium (containing 0.1 mg/ml ampicillin) with a loop-full of stock culture. This was grown overnight at $37^{\circ}C$ with constant shaking. One milliliter of the overnight-grown culture was then inoculated into 500 ml of LB medium (containing 0.1 mg/ml ampicillin) and grown overnight at $37^{\circ}C$ with constant shaking.

When it was necessary to amplify the plasmid in **E. coli,** the overnight incubation was interrupted by addition of 0.5 ml of 170 mg/ml of chloramphenicol into the 500 ml culture after 2-3 hours of incubation.

(V) Isolation and purification of Achlya genomic DNA

Buffers and enzymes

Buffer A contained 0.05 M K-maleate and 0.5 M $MgSO_4$, pH 5.8. Buffer B was buffer A plus 1% (w/v) driselase*. Lysis

buffer contained 0.1 M NaCl, 0.1 M Na_2EDTA, 50 mM Tris-Cl, pH 8.0 and 1% (w/v) sarkosyl.

Stock solution (10 mg/ml) of bacterial proteinase was prepared by dissolving proteinase in 50 mM Tris-Cl, pH 8.0 solution and heating it in 37°C waterbath for 1 hour. Stock solution of DNase-free RNase was prepared by dissolving pancreatic RNase (RNase A) at a concentration of 10 mg/ml in 10 mM Tris-Cl, pH 7.5 and 15 mM NaCl solution, and heating it for 15 minutes at 100°C.

* dresilase (cellulase) was from Kyowa Hakko Kogyo Co.Ltd. Tokyo. Japan.

Protocol

Germling grown in G_2Y medium were collected by filtration using Millipore filters (10 µm pore size) and washed with approximately 200 ml of distilled water. Germling mat, obtained from 500 ml medium, was suspended in 20 ml of buffer B and incubated in 37°C waterbath for approximately one hour with gentle shaking. The suspension was centrifuged at 2,000 x g for 5 minutes, the supernatant was discarded and the pellet resuspended in 10 ml of buffer A. The suspension was centrifuged at 2,000 x g for 5 minutes, supernatant discarded and the pellet suspended in 8 ml of lysis buffer. DNase-free RNase (final concentration: 100 µg/ml) was added to the suspension and followed by

incubation in $37^{o}C$ waterbath for approximately 3 hours with gentle shaking. At this point, concentrated bacterial proteinase was added to the suspension (final concentration: 100 µg/ml) and incubated at 37° with gentle shaking for another 3 hours. After incubation, about 1 g of CsCl and 25 µl of 25% EtBr were added to every ml of the lysate. CsCl crystal was dissolved by carefully inverting the tube sealed with parafilm.

The suspension was then transferred into 12 ml Beckman ultracentrifuge quick-seal tubes. The tubes were centrifuged in a Beckman type L8-80 ultracentrifuge using Beckman 80 Ti rotor at 60,000 rpm for 20 hours at $14^{o}C$.

After centrifugation, the DNA band was carefully removed by a hypodermic (G 20) needle. Ethidium bromide in the DNA eluate was removed by extraction with CsCl-saturated isopropanol, and the DNA solution was then dialyzed overnight with two changes against 0.5 l of TE buffer (10 mM Tris-Cl, 1 mM Na_2EDTA, pH 7.6).

The concentration of DNA solution was determined spectrophotometrically based on standard rule: 1 unit of $O.D._{260}$ = 50 µg/ml of DNA. The DNA was stored in autoclaved Eppendorf tubes at $4^{o}C$.

Comment:

Many other methods were tried. Different methods gave different yield and degree of purity. The method given above

gave the best yield and purity.

(VI) Large-scale isolation and purification of *E. coli* plasmid

Two different procedures were employed:

(1) Procedure employing CsCl-gradient centrifugation

Buffers

Sucrose buffer contained 15% (w/v) sucrose, 50 mM Tris-Cl, pH 8.0, 50 mM Na_2EDTA, pH 8.0. Triton buffer contained 50 mM Tris-Cl, pH 8.0, 50 mM Na_2EDTA, pH 8.0, and 0.4% (v/v) Triton X 100. TE buffer contained 10 mM Tris-Cl, pH 7.5, and 1 mM Na_2EDTA.

Protocol

Large-scale overnight **E. coli** culture was centrifuged in 0.5 l plastic centrifuge bottles at 8,000 x g for 10 min. Pelleted cells from 1 liter culture were resuspended in 4 ml of sucrose buffer and transferred to a screw-capped centrifuge tube. Approximately 50 mg of lysozyme were added to the suspension, mixed by vortexing and incubated on ice for 5 min. Six milliliter of Triton buffer was added and mixed by rocking and rolling by hand slowly for about 5 min. The cells lysed and the lysate became viscous. Whenever the cells did not lyse, the suspension was heated in a $65^{o}C$

waterbath for 15-30 min until the cells lysed. Once the cells lysed, the suspension was centrifuged at 30,000 x g for 40-45 min. The aggregated bacterial genomic DNA sedimented forming a packed white pellet and the lighter plasmid DNA remained in the supernatant. Whenever the pellet was not hard, centrifugation was repeated. The supernatant was transferred into a separate container and kept on ice throughout. One gram of CsCl crystal was added per ml of supernatant and dissolved by gentle agitation. Two hundred microliters of 2% EtBr was added for every 8 ml of the mixture. After thorough mixing, the solution was transferred into 12 ml capacity Beckman ultracentrifuge quick-seal tubes. The tubes were centrifuged in a Beckman type L8-80 ultracentrifuge using Beckman 80 Ti rotor at 60,000 rpm for 16-20 hours at 14°C.

When ultracentrifugation was completed, two DNA bands appeared in the CsCl gradient. The plasmid, which appeared as the bottom of the two bands, was removed carefully by a hypodermic syringe fitted with a size 20 needle. It was transferred into a conical glass centrifuge tube. To remove EtBr, equal volume of the CsCl-saturated isopropanol was added to the plasmid. It was mixed gently by tapping with a fingertip and allowed to sit until the two phases were separated. Using a pasteur pipette, the top phase was removed. This process was repeated until colour was

practically gone from the plasmid aqueous phase. The plasmid was dialyzed overnight with two changes against 0.5 l of TE buffer.

The concentration of plasmid DNA was determined spectrophotometrically with the standard rule: 1 unit of $O.D._{260}$ = 50 μg/ml of DNA. The plasmid was stored in autoclaved Eppendorf tubes at $4^{o}C$.

(2) Procedure employing NaOH/SDS

Buffers and solutions

Lysis buffer consisted of 2 mg/ml lysozyme, 25 mM Tris-base, 10 mM Na_2EDTA, and 50 mM glucose. NaOH/SDS buffer contained 0.2 M NaOH and 1% (w/v) SDS. TAES buffer contained 40 mM Tris-base, 0.1 M Na acetate, 1 mM Na_2EDTA, and 0.1% (w/v) SDS. Ribonuclease A (2 mg/ml) in 5 mM Tris-Cl, pH 8.0, which had previously been treated at $100^{o}C$ for 10 min.

Protocol

One liter overnight-grown **E. coli** culture was centrifuged in plastic centrifuge bottles. The pellet was suspended in 40 ml of lysis buffer and kept on ice for 30 min. Following the incubation, 80 ml of NaOH/SDS buffer was added to the suspension and mixed gently by inversions. Incubation on ice was resumed for 5 min before the suspension was transferred to glass centrifuge tubes. Sixty milliliter

of 3 M sodium acetate (pH 4.8) was mixed with the suspension and it was further incubated on ice for 60 min with occasional inversions. To separate plasmid from genomic DNA, the suspension was spun at 12,000 x g for 10 min; plasmid remained in the supernatant while genomic DNA pelleted. Two volumes of cold ethanol were mixed with the supernatant and stored at -80°C for 30 min. The precipitated plasmid DNA was then pelleted by centrifugation for 5 min at 12,000 x g. The pellet was saved and extracted with 40 ml of TAES buffer and 40 ml of phenol:chloroform (1:1) mixture. The phenol extraction was repeated, each time this was done with great caution not to include the interphase. The aqueous phases were pooled. Two volumes of cold ethanol were added to it and stored at -20°C overnight.

The precipitate was recovered the next day by centrifugation at 12,000 x g for 5 min. The pellet was suspended in 10 ml of distilled water and 4 ml of 1 M sodium acetate (pH 8.0). Two volumes of cold ethanol were added to the suspension, followed by freezing at -80°C for 30 min. The precipitate was removed by centrifugation, resuspended and reprecipitated.

To remove any RNA contaminant, the pellet was dissolved in 4 ml of water and 0.2 ml of ribonuclease A solution, followed by incubation at 37°C for 30 min. One volume of ethanol and sodium acetate, pH 6.0 (final concentration of 75

mM) were mixed with the suspension. It was incubated at room temperature for 10 min followed by centrifugation. This step was repeated 3-4 times and the trace of ethanol was removed by evaporation in a jet of air.

The pellet was dissolved in 2 ml of 1 mM EDTA and stored frozen in Eppendorf tubes. The concentration of plasmid DNA was determined spectrophotometrically.

(VII) Restriction-endonuclease digestion of DNA

Restriction endonuclease enzymes and buffers

Restriction endonuclease enzymes were purchased from BRL, Boehringer, and P.L. Biochemical companies.

Buffers used for restriction enzyme digestion were of two groups. The first group of buffers consisted of buffers described according to the salt concentration they contained. Ten times concentration of low salt buffer contained 0.1 M Tris-Cl (pH 7.4), 0.1 M $MgSO_4$ and 10 mM DTT. Ten times concentration of medium salt buffer contained 0.5 M NaCl, 0.1 M Tris-Cl (pH 7.4), 0.1 M $MgSO_4$ and 10 mM DTT. And 10 x concentration of high salt buffer contained 1.0 M NaCl, 0.5 M Tris-Cl (pH 7.4) and 0.1 M $MgSO_4$. The second group of buffers were prepared as specified by the enzyme suppliers. Each buffer was specific for only one enzyme.

Reaction mixture for DNA digestion

All restriction enzyme digestions of DNA were carried out in Eppendorf tubes at 37°C.

Each reaction mixture consisted of 1 x concentration buffer, restriction enzyme (1-3 units per µg of DNA) and DNA.

The quantity of DNA digested in each tube varied. For optimum digestion, the DNA solution added did not exceed half of the total volume of the reaction mixture. In a few cases, 0.1% final concentration of bovine serum albumin was added into the reaction mixture.

The time allowed for digestion varied depending on the purpose it served. Partial digestion of **Achlya** DNA was carried out for 4 to 5 hours and complete digestion for 18 to 20 hours.

Reactions were terminated by the addition of 0.25 volume of a solution composed of 4 M urea, 50% (w/v) sucrose, 50 mM Na_2EDTA, 0.1% (w/v) bromophenol blue, pH 7.0.

(VIII) Gel electrophoresis

Buffer

Tris-borate running buffer contained 89 mM Tris-base, 89 mM boric acid, 2.5 mM Na_2EDTA, pH 8.3.

Protocol

Submarine horizontal gel was prepared as follows: The agarose gel (0.7-1.0%) was prepared in 175 ml to 200 ml of running buffer by boiling. Boiled agarose was cooled to about 60°C before casting into a gel-supporting tray (which had previously been sealed both sides using masking tape). Depending on the volume of wells desired, different sizes of gel comb were used.

The gel was completely submerged (about 1-2 mm) underneath 0.5 x electrophoresis running buffer which contained 0.5 µg/ml of EtBr. One volt/cm (of gel length) was applied for 16 to 20 hours.

(IX) Photography

Ethidium bromide-stained gels illuminated by incident U.V. light were photographed using Polaroid film (type 667). A red wratten filter was used to reduce background light and enhance fluorescence produced by U.V. light on EtBr-stained DNA. Photographic exposure time varied from 30-90 seconds.

(X) Southern transfer

The technique of the transfer of DNA fragments, that have been separated according to size by electrophoresis through an agarose gel, to a filter membrane has been

described by Southern (1975).

Two slightly modified methods of blotting were used for two different kinds of membrane filter namely: nitrocellulose filter and Gene screen / Gene screen plus.

(1) Transfer of DNA from agarose gel to nitrocellulose paper

The method followed for the transfer was as given in "Molecular cloning, A laboratory manual" by Maniatis et al. (1982).

(2) Transfer of DNA from agarose gel to Gene screen / Gene screen plus

The protocol for capillary transfer of DNA to Gene screen / Gene screen plus were as supplied by New England Nuclear Co.

(XI) Isolation and purification of oncogene DNA insert from oncogene-carrying plasmids

A large amount of plasmid DNA was digested with the pertinent restriction endonuclease(s) that specifically cut in a manner that excised the oncogene DNA insert from the vector plasmid pBR 322.

The DNA sample was run on a suitable horizontal gel to

separate oncogene DNA fragments from pBR 322 fragments. The desired DNA band was cut out, using a scalpel, from the gel slab. About 0.5 cm more around the sides in the gel, where the band once was, was removed to make a trough larger than the band. A sheet of dialysis tubing was placed over the trough with enough to cover at least 1 cm over all edges of the trough. The excised DNA band was placed within this dialysis sheet in the trough and 100-300 µl of running buffer added. The volume of the running buffer was reduced so that only half the thickness of the gel was immersed. The gel was electrophoresed at 150 volts for 1-2 hours until all the DNA fragment was electro-eluted from the gel strip. The buffer was withdrawn from the trough and about 200 µl of new buffer was added before the current was reversed for 2-3 min. The buffer eluates were pooled.

To remove EtBr from the DNA, three volumes of water-saturated isoamyl alcohol were mixed with the buffer collected, vortexed and centrifuged. The isoamyl alcohol phase was removed and the step was repeated 5-10 times. Tris-EDTA buffer-saturated phenol was used to remove traces of protein from the DNA preparation, sodium acetate was added to 0.3 M final concentration and two volumes of chilled absolute ethanol. The DNA was precipitated overnight at -20°C and collected by centrifugation in an Eppendorf microcentrifuge. The pellet was lyophilized to remove traces

of ethanol, dissolved in a small amount of sterile distilled water and stored at -4°C.

In order to determine the DNA concentration, five microliters of DNA was spotted onto a petri plate containing EtBr-incorporated 1% agarose. Along with the sample, equal volumes of other DNA samples of known concentration were also spotted. The concentration of the recovered DNA was determined by comparing their intensities under U.V. light.

(XII) Nick translation of DNA fragment or whole plasmid

Buffers, enzymes and radioactive nucleotides

The nick translation kit supplied by Amersham corp. was used. ^{32}P-labelled-dCTP and ^{32}P-labelled-dTTP were purchased from New England Co. Stop buffer contained 0.02 M Na_2EDTA, 2 mg/ml sonicated salmon sperm DNA and 0.2% (w/v) SDS.

Protocol

A typical reaction mixture of 30 µl contained 70 µCi of ^{32}P-labelled-dCTP, 70 µCi of ^{32}P-labelled-dTTP, 250 ng of DNA, 0.5 µl of 0.1 mM dCTP, 0.5 µl of 0.1 mM dTTP, 5.0 µl of 0.1 mM combined dATP and dGTP, and 3.0 µl of enzymes (10 µl aliquot contained 5 units DNA polymerase I and 100 pg DNase I in a buffer containing Tris-Cl, pH 7.5, $MgCl_2$, glycerol and bovine serum albumin).

The reaction was carried out in a sterile Eppendorf tube at 14°C for 1.5 hours. The reaction was terminated, by adding 20 µl of stop buffer plus 10 µl of 4 µg/µl yeast t-RNA, and then heated for 15 min at 68°C.

A 5 ml G-50 sephadex column was used to separate the labelled DNA from free radionucleotide using TE buffer (10 mM Tris-Cl, 1 mM Na_2EDTA, pH 8.0) as eluent. The reaction mixture was passed through the chromatography column and the first radioactive peak, which contained nick-translated DNA, was eluted into an Eppendorf tube. A portable Geiger counter was used to monitor the radioactivity.

Radioactive count

Five microliters of sample were taken out from the eluate for radioactivity counting. Five microliters of 10 mg/ml bovine serum albumin plus 100 µl of 20% TCA were added to the sample. After incubation on ice for 10-15 min, the mixture was filtered through a 0.45 µm Millipore filter and washed with 20% TCA. The filter was placed into a scintillation vial containing 10 ml of toluene scintillation fluid and radioactivity was counted in a Beckman LS 230 liquid scintillation spectrometer.

Total count of radioactivity was calculated as follows:

For example: radioactive count of 5 µl eluate = 41,000 cpm

total volume of eluate = 1.0 ml

DNA nick translated = 250 ng

Total count for 1 μg of DNA :

$$\frac{1000\ \mu l}{5\ \mu l} \times \frac{1000\ ng}{250\ ng} \times 41{,}000\ cpm = 33.6 \times 10^{6}\ cpm/\mu g\ of\ DNA$$

(XIII) Hybridization of nick-translated DNA to membrane-bound DNA

(1) Protocol I

Buffers and solutions

One time SCP buffer was made up of 0.1 M NaCl, 30 mM Na_2HPO_4 and 1 mM Na_2EDTA, pH 6.2. One time SSC buffer contained 0.15 M NaCl and 0.015 M Na acetate, pH 7.0. Denhardt´s solution contained 0.2 g bovine serum albumin type V, 0.2 g polyvinyl-pyrrolidone 360 and 0.2 g Ficoll 400 per liter of water.

Prehybridization solution contained 6.6 x SCP, 1% (w/v) sarkosyl, 0.1 mg/ml of sonicated salmon sperm DNA, and 10 x Denhardt´s solution. The solution was degassed before use.

Protocols for hybridization and washing

For each hybridization, 150 ml of prehybridization solution was prepared. A hundred and thirty milliliters was used for prehybridization incubation of the nitrocellulose or Gene screen / Gene screen plus filters, while the remaining

20 ml was used for the hybridization incubation.

The membrane was first soaked in distilled water, and then in 4.4 x SSC solution at room temperature for a few minutes to ensure total wetness of the membrane. The membrane was transferred into a plastic bag with 130 ml of prehybridization solution. All air bubbles were removed and the bag was heat sealed. The entire plastic bag was then immersed in a 55°C shaking waterbath for 3 hours or more (for nitrocellulose membrane), or 6 hours or more (for Gene screen / Gene screen plus).

To the remaining 20 ml of the prehybridization solution, 2.0 g of dextran sulfate (M.W. 500,000) were added and dissolved by heating. Again a thorough degassing was done to remove all air bubbles.

Following prehybridization, the prehybridization solution was replaced by the dextran sulfate-containing hybridization solution. The nick-translated DNA was denatured by boiling for 10 min and rapidly chilled in ice for 10 min. The probe was pour into the plastic bag, bubbles removed and the bag resealed. Hybridization was done in a 55°C shaking waterbath for a period of 24-36 hours.

Washings were done 2 x 15 min in 6.6 x SCP and 1% (w/v) sarkosyl, and 2 x 90 min in 1 x SCP and 1% (w/v) sarkosyl. Each washing was done in a 55°C shaking waterbath.

(2) Protocol II

Solutions

Prehybridization and hybridization solutions contained 6 x SSC, 4 x Denhardt´s solution, 0.1% (w/v) SDS, and 1 mg/ml of sonicated salmon sperm DNA.

Protocols for hybridization and washing

The prehybridization and hybridization processes were much the same as protocol I. The solution used for prehybridization was 30 ml, and 10 ml for hybridization.

Washings were done 2 x 20 min in 2 x SSC, 0.1% (w/v) SDS solution; 1 x at room temperature and 1 x at 55°C in a shaking waterbath. Twice 20 min in 1 x SSC, 0.1% (w/v) SDS solution; 1 x at room temperature and 1 x at 55°C in a shaking waterbath. Twice 20 min in 0.5 x SSC and 0.1% (w/v) SDS solution, again with similar alternating room and 55°C temperature

(3) Protocol III

This protocol was used only for Gene screen plus membrane. The method was as outlined by New England Nuclear Co. Gene screen plus product, catalog No. NEF-976 (January 1984).

(XIV) Autoradiography

Following the washing process, nitrocellulose or Gene screen / Gene screen plus membrane was placed between two sheets of saran wrap for autoradiography purposes. The X-ray film used was Kodak X-Omat RP. A Kodak X-Omatic regular intensifying screen or, in some cases, an X-ray AQ exposure holder was used.

The length of exposure depended on the radioactivity. Generally, it was for 1 to 4 days.

R E S U L T S

(I) Isolation and purification of Achlya genomic DNA

Isolation and purification of **Achlya** genomic DNA as described in Materials and Methods was done several times to obtain a sufficient supply of **Achlya** DNA.

The DNA band following CsCl-gradient ultracentrifugation, which could be seen under U.V. light illumination, varied in thickness in different isolations.

Different batches of DNA isolated varied in concentration from 100 to 450 µg/ml. A low-concentration DNA solution was concentrated by ethanol precipitation and dissolved in a small amount of water prior to use.

The purity of the DNA was tested enzymatically. Small aliquots of DNA sample were subjected to digestions with DNase or RNase, and then run on agarose gel. Figure 1 shows that when the sample were treated with nothing (well 1) or RNase (well 2) the DNA bands appeared. When the sample was treated with DNase (well 3), the DNA band disappeared. The DNA intensity was comparable for wells 1 and 2; this showed that the DNA was pure and essentially free of RNA contaminant. Figure 1 also shows the approximate size of the DNA isolated, which was of 23.0 kb. or larger.

Figure 1.

Gel electrophoresis of samples of the **Achlya** genomic DNA. Four microgram samples of the DNA were digested overnight with 10 μg of DNase-free RNase (well 2), with 2.5 μg of DNase (well 3), and with no enzyme as a control (well 1). The samples were run, after overnight digestion, on 1% agarose gel for 16 hours at 36 volts. The well on the leftmost side contained standard λ-Hind III-cut DNA markers; arrows and numbers indicate the size of each band in kilobasepairs.

Figure 2.

Gel electrophoresis of samples of the pbC-N1 plasmid DNA. Two microgram samples of plasmid DNA were digested with no enzyme as a control (well 1), Bam HI (well 2), Eco RI (well 3), Sal I (well 4) overnight. Well 5 contained standard λ-Hind III-digested DNA markers; arrows and numbers indicate the size of each band in kilobasepairs.

Figure 3.

Gel electrophoresis of samples of the pT24-C3 plasmid DNA. Two microgram samples of the plasmid DNA were digested with no enzyme as a control (well 1), Bam HI (well 2), Sal I (well 3), Bam HI and Eco RI (well 4) overnight. Well 5 contained standard λ-Hind III-digested DNA markers; arrows and numbers indicate the size of each band in kilobasepairs.

Figure 4.

Gel electrophoresis of samples of the pHB-11 plasmid DNA. Approximately 5 μg samples of the plasmid DNA were digested with no enzyme as a control (well 1), Eco RI (well 2), Eco RI λ-Hind III-digested DNA markers; arrows and alphabets indicate the positions of the standard DNA bands. Their sizes, in descending order, are 23.1, 9.5, 6.7, 4.3, 2.3, 2.0 kilobasepairs.

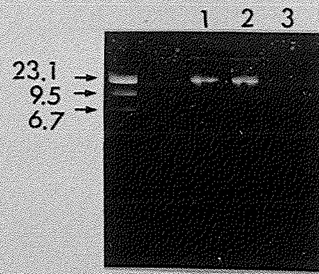

Fig. 1

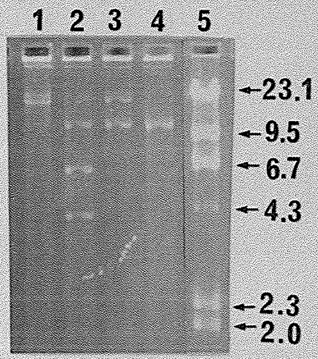

Fig. 2

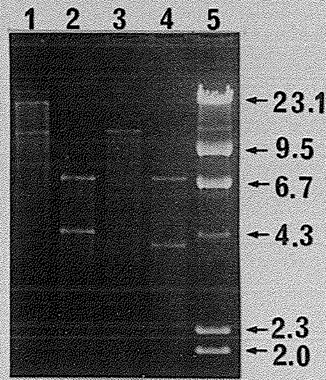

Fig. 3

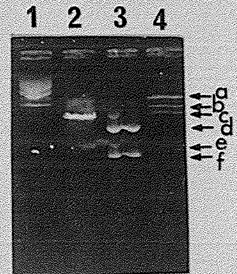

Fig. 4

(II)(i) Isolation and purification of plasmid DNAs

Two methods of isolation and purification were employed. The method employing CsCl-gradient ultracentrifugation gave a better yield as well as purity.

Plasmid pbC-N1 was isolated using the NaOH/SDS method. From one liter of **E. coli** culture, about 400 μg of plasmid DNA was obtained.

Characterization of the pbC-N1 plasmid by electrophoresis (figure 2) showed that the plasmid DNA isolated was essentially pure and free of other DNA or RNA contaminants. When the pbC-N1 was digested with no enzyme (well 1), one DNA band representing the circular plasmid DNA appeared. The digestion of the pbC-N1 with Bam HI (well 2) produced two DNA bands representing the 6.4 kb. human c-Ha-ras proto-oncogene fragment and the 4.3 kb. pBR 322 fragment. A band at the position of 10.7 kb. which represented the linear pbC-N1 also appeared; this indicated that the digestion was somewhat incomplete. The pbC-N1 was also digested with enzymes Eco RI (well 3) and Sal I (well 4) which cut only once into the plasmid. When run on the gel, both samples produced DNA bands at 10.7 kb. position, the intact size of linear pbC-N1.

Plasmid pT24-C3 was isolated by CsCl-gradient ultracentrifugation. A yield of roughly 1 mg was purified

from one liter of **E. coli** culture.

Figure 3 shows the characterization of the pT24-C3 plasmid DNA by electrophoresis. As shown in the figure, when pT24-C3 was digested with no enzyme (well 1), two DNA bands were observed. The DNA band at the higher position represented the undigested plasmid DNA, while the DNA band at the lower position represented the linear pT24-C3. The digestion of the pT24-C3 with Bam HI (well 2) separated the 6.6 kb. c-Ha-ras oncogene fragment from the 4.3 kb. pBR 322 fragment forming two DNA bands at those positions. The pT24-C3 was linearized when digested with Sal I (well 3) and cut into three fragments when digested with a combination of Bam HI and Eco RI (well 4). This latter digestion produced the 6.6 kb. c-Ha-ras fragment, the 4.0 kb. portion of the pBR 322 and the 0.3 kb. portion of the pBR 322 which was at a lower position out of the photographic field.

Plasmid pHB-11 was purified by CsCl-gradient ultracentrifugation method. About 800 µg of plasmid DNA was obtained from one liter of culture.

The plasmid isolated was characterized by electrophoresis as shown in figure 4. The pHB-11 digested with no enzyme (well 1) when run on the gel produced two DNA bands. They represented the coiled-undigested and the non coiled-undigested pHB-11. The digestion with Eco RI (well 2) linearized the pHB-11 forming a 6.5 kb. DNA band, while the

digestion with Eco RI and Bam HI (well 3) separated the 2.2 kb. v-Ha-ras fragment from the 4.3 kb. pBR 322.

(ii) Recovery and purification of DNA insert

Recovery and purification of DNA fragments from DNA bands separated on agarose gels, as described in the Materials and Methods, give yields varying from roughly 2% to 10%. Each recovery and purification was tested out for the presence and purity of the DNA fragment by means of gel electrophoresis.

Figure 5 shows the gel electrophoresis of a sample of the v-Ha-ras DNA insert recovered from an agarose gel. The 2.2 kb v-Ha-ras oncogene migrated to its appropriate position.

Since the amount of DNA recovered was usually in small quantity, the concentration of the DNA was determined roughly by comparing its fluorescence intensity to the intensities of a set of standard DNA as shown in figure 6.

Comment:

The oncogene DNA insert recovered was initially dissolved in TE buffer (10 mM Tris-Cl, 1 mM Na_2EDTA). This proved later to cause problems in nick translation. I decided then to dissolve the DNA fragment recovered in

Figure 5.

Gel electrophoresis of a sample of the v-Ha-ras DNA insert recovered from agarose gel. Ten microliters sample of the 2.2 kb. DNA fragment recovered from plasmid pHB-11 was run on agarose gel (well 2). Well 1 contained standard λ-Hind III-digested DNA markers; arrows and numbers indicate the sizes of DNA bands in kilobasepairs.

Figure 6.

Estimation of DNA concentration by comparison of their fluorescence intensities. Five microliter samples of DNA solution were spotted onto an agarose gel containing ethidium bromide. Numbers 1 to 10 were spots of λ DNA with known concentrations. Their concentrations range from 25 to 250 μg/ml with 25 μg/ml increment. Five microliters of v-Ha-ras DNA solution was spotted above "v-ras".

Figure 7.

Gel electrophoresis of samples of the **Achlya** genomic DNA digested with various restriction endonucleases. Twenty microgram samples of the DNA were digested with Eco RI (2), Xba I (3), Pst I (4), Sau 3A (5), Bam HI (6), and Hind III (7) for 16 hours. The samples were run on 0.7% agarose gel for 15 hours at 30 volts. Well 1 contained standard λ-Hind III-digested DNA markers; arrows and numbers indicate the sizes of DNA bands in kilobasepairs.

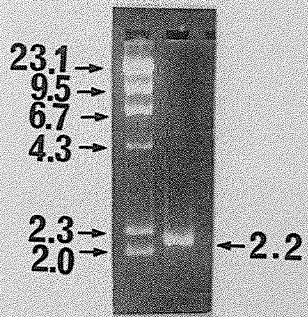

Fig.5

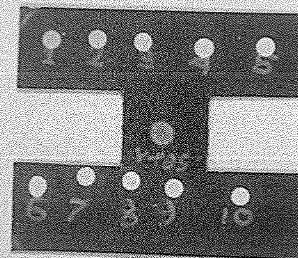

Fig.6

Fig. 7

deionized distilled water.

(III) Gel electrophoresis and Southern blotting

To probe **Achlya** genomic DNA for the presence of ras oncogene-related sequence(s), the **Achlya** DNA was digested with various restriction endonuclease, separated based on size by electrophoresis, denatured and transferred onto a membrane filter for hybridization analysis.

A complete or partial restriction enzyme-digested **Achlya** DNA when run on agarose gels showed typical banding on a smear background.

Figure 7 shows the electrophoresis of the **Achlya** genomic DNA after being digested with restriction enzymes. The **Achlya** genomic DNA digested with enzymes which recognize six nucleotide base sequences (Eco RI, Xba I, Pst I, Bam HI, and Hind III) produced larger DNA fragments. This appeared as smears located at positions closer to the top of the gel (wells 2, 3, 4, 6 and 7). The **Achlya** DNA digested with enzyme which recognizes four nucleotide base sequences (Sau 3A) produced smaller DNA fragments. This appeared as smear starting at a lower position (well 5).

(IV) Nick translation of DNA probe

The ras oncogene DNA, used to probe for the presence of ras-related sequence(s) in the **Achlya** genomic DNA, was first labelled with ^{32}P-containing nucleotides using a nick translation technique described in the Materials and Methods.

The total counts of radioactivity of the nick-translated DNA probe vary from 3 x 10^7 cpm/µg of DNA to 1 x 10^8 cpm/µg of DNA.

(V) Hybridization analysis of Achlya DNA

Denatured **Achlya** DNA blotted onto a membrane filter was hybridized to denatured radioactive-labelled ras oncogene probe.

The presence of sequence(s) homologous to the ras oncogene probe appeared as radioactive band(s) in autoradiogram.

(i) c-Ha-ras-carrying plasmid as a probe

Southern blot analysis of **Achlya** DNA digested with Hind III, Xba I and Eco RI, and probed with c-Ha-ras-carrying plasmid are shown in figures 9, 10 and 11. The ras oncogene probe hybridized to Hind III-digested **Achlya** DNA fragments of approximately 3.0 kb. and 1.5 kb. sizes (figure 9), and of

approximately 8.5 kb. sizes (figures 10 and 11). The ras probe also hybridized to Xba I-digested **Achlya** DNA fragment of approximately 8.0 kb. size (figures 9, 10 and 11), and to Eco RI-digested **Achlya** DNA fragments of approximately 15.0, 6.0 and 0.5 kb. in size (figure 9).

The results above are summarized in table 2.

It was observed that, when the pbC-N1 / pT24-C3 was used as a probe in the hybridization analysis, there was hybridization between the probe and the 23.1 kb., 6.6 kb., and 4.3 kb. fragments of the λ-Hind III-digested DNA marker, as shown in figure 8.

Figures	Restriction enzymes	Approximate sizes of hybridization bands
9	Hind III	3.0 kb. 1.5 kb.
	Xba I	8.0 kb.
	Eco RI	15.0 kb. 6.0 kb. 0.5 kb.
10	Hind III	8.5 kb.
	Xba I	8.0 kb.
11	Hind III	8.5 kb.
	Xba I	8.0 kb.

Table 2.

Figure 8.

The hybridization between radioactive-labelled pbC-Nl probe and λ-Hind III-digested DNA markers. Arrows and numbers indicate the locations and sizes of the hybridization bands in kilobasepairs.

Figure 9.

Southern blot analysis of restriction endonuclease-digested, high-M.W. **Achlya** genomic DNA. Ten microgram samples of the DNA were digested with Hind III (1), Xba I (2), and Eco RI (3) for 18 hours, electrophoresed, blotted and hybridized (55°C hybridization and washing conditions) to radioactive-labelled pbC-Nl probe. Arrows and numbers indicate the locations and sizes of the hybridization bands in kilobasepairs.

Figure 10.

Southern blot analysis of restriction endonuclease-digested, high-M.W. **Achlya** genomic DNA. Five microgram samples of the DNA were digested with Hind III (1), Xba I (2) for 12 hours, electrophoresed, blotted and hybridized (55°C hybridization and washing conditions) to radioactive-labelled pbC-Nl probe. Arrows and numbers indicate the locations and sizes of the hybridization bands in kilobasepairs.

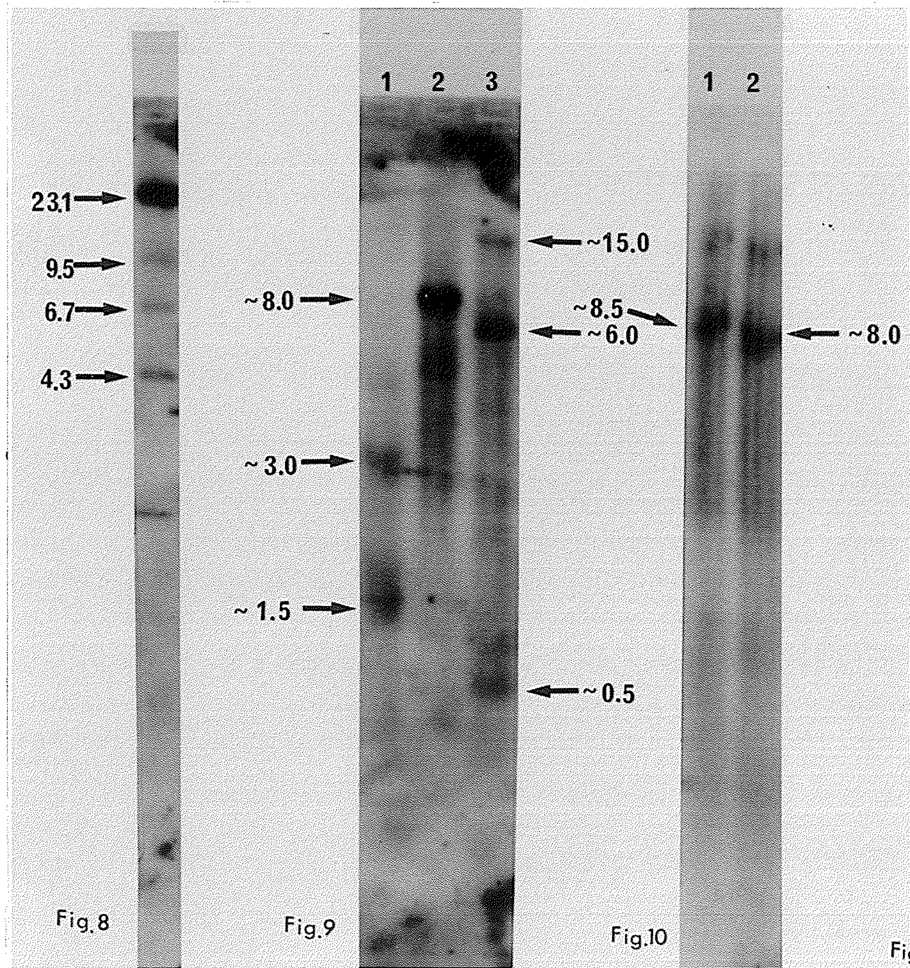
23.1
9.5
6.7
4.3
Fig. 8
1 2 3
~8.0
~3.0
~1.5
~15.0
~6.0
~0.5
Fig.9
~8.5
1 2
~8.0
Fig.10

Figure 11.

Southern blot analysis of restriction endonuclease-digested, high-M.W. **Achlya** genomic DNA. Five microgram samples of the DNA were digested with Hind III (1), and Xba I (2) for 2 hours, electrophoresed, blotted and hybridized ($65^{o}C$ hybridization and washing conditions) to radioactive-labelled pbC-N1 probe. Arrows and numbers indicate the locations and sizes of the hybridization bands in kilobasepairs.

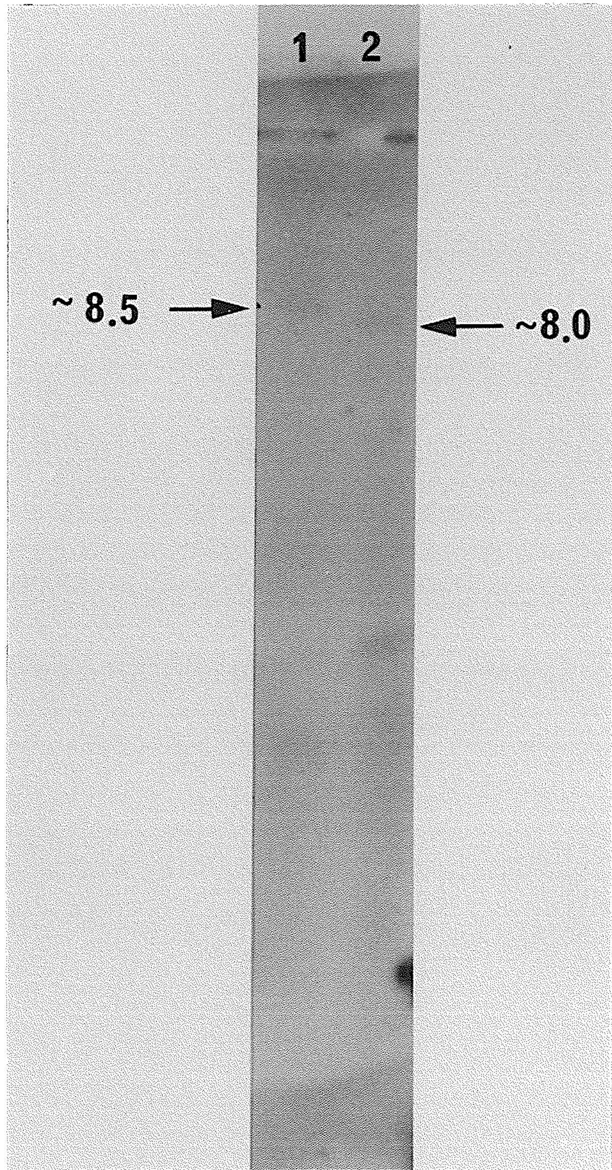

Figure 11

The analysis in figures 9 and 10 were carried out in relaxed hybridization conditions i.e. 55°C hybridization and 55°C washing. The analysis in figure 11 was carried out in a more stringent hybridization conditions i.e. 65°C hybridization and 65°C washing.

(ii) v-Ha-ras oncogene as a probe

Southern blot analysis of **Achlya** genomic DNA digested with Pst I, Bam HI and Xho I, and probed with v-Ha-ras oncogene are shown in 12 and 13. When probed with v-Ha-ras oncogene, two fragments of DNA hybridized to the probe in Pst I-digested **Achlya** DNA. They are of approximately 9.0 and 5.0 kb. in size. One hybridization band was detected in Bam HI-digested **Achlya** DNA, corresponding to approximately 3.0 kb. in size. Another band approximately 2.2 kb. in size was detected in Xho I-digested **Achlya** DNA.

The results above are summarized in table 3.

Figures	Restriction enzymes	Approximate sizes of hybridization bands
12	Pst I	9.0 kb. 5.0 kb.
13	Bam HI	3.0 kb.
	Xho I	2.2 kb.

Table 3.

Figure 12.

Southern blot analysis of restriction endonuclease-digested, high-M.W. **Achlya** genomic DNA. A ten microgram sample of the DNA was digested with Pst I for 5 hours, electrophoresed, blotted and hybridized ($55^{o}C$ hybridization and washing conditions) to radioactive-labelled 2.2 kb. v-Ha-ras probe. Arrows and numbers indicate the locations and sizes of the hybridization bands in kilobasepairs.

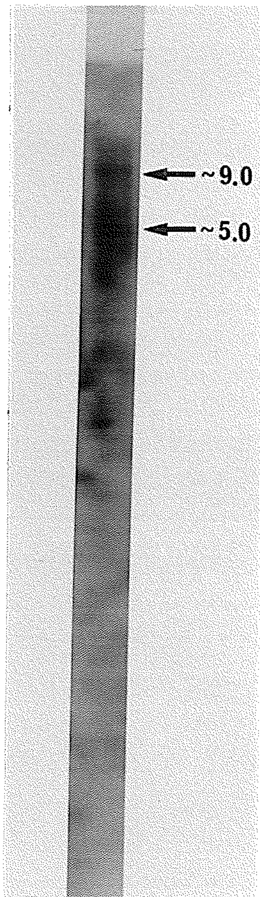

Figure 12

Figure 13.

Southern blot analysis of restriction endonuclease-digested, high-M.W. **Achlya** genomic DNA. Twenty microgram samples of the DNA were digested with Bam HI (1), and Xho I (2) for 16 hours, electrophoresed, blotted and hybridized ($55^{o}C$ hybridization and washing conditions) to the radioactive-labelled 2.2 kb. v-Ha-ras probe. Arrows and numbers indicate the locations and sizes of the hybridization bands in kilobasepairs.

Figure 14.

Southern blot analysis of restriction endonuclease-digested rat DNA. Five microgram samples of the DNA were digested with Eco RI (1), Xba I (2), and Xho I (3) for 3.5 hours, electrophoresed, blotted and hybridized ($55^{o}C$ hybridization and washing conditions) to the radioactive-labelled 2.2 kb. v-Ha-ras probe. Arrows and numbers indicate the locations and sizes of the hybridization bands in kilobasepairs.

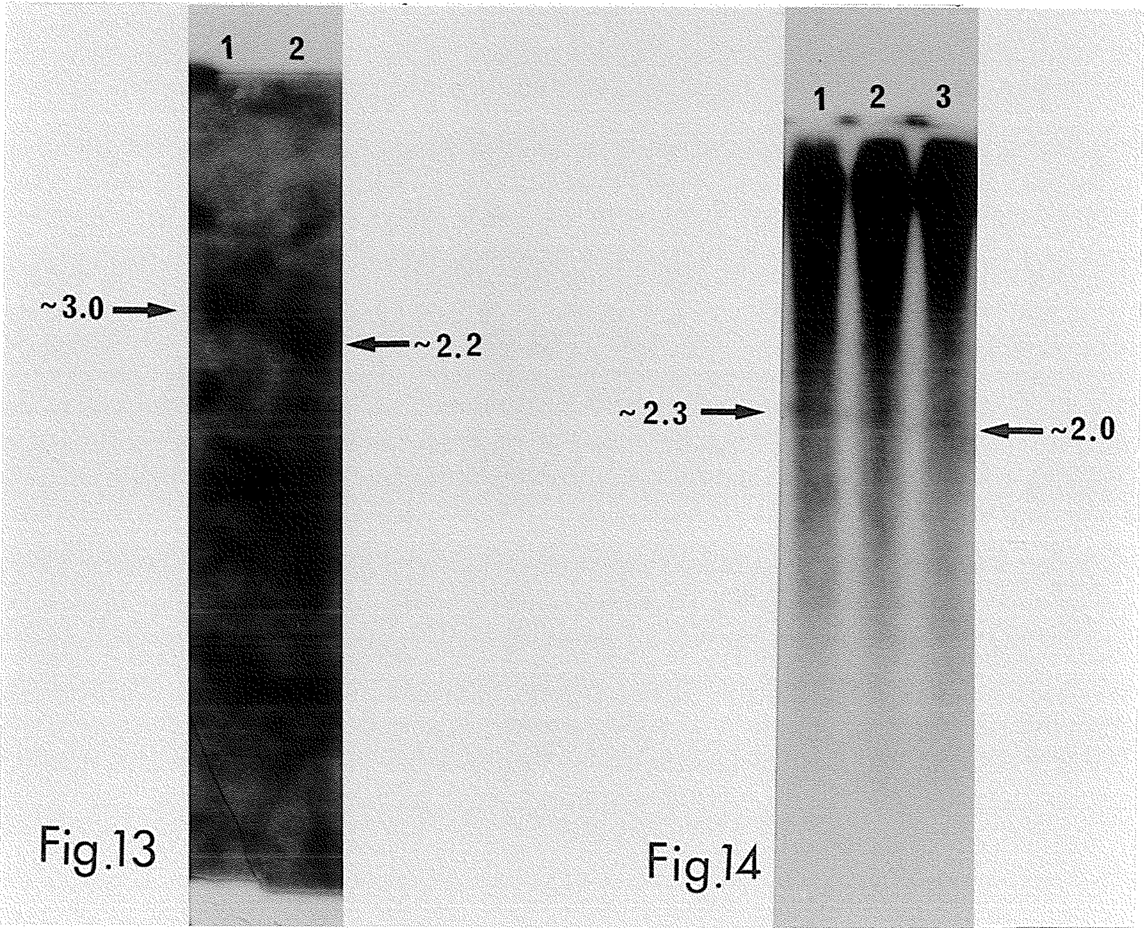

Fig.13

Fig.14

The analysis in figures 12 and 13 were carried out in relaxed hybridization conditions i.e. 55°C hybridization and 55°C washing.

Comment:

The rather dubious hybridization bands shown in figure 13 were considered valuable because they were repeated in other analysis which are not shown here. The problem of high background in this particular Southern blot analysis may be attributed to the insufficient prehybridization "coating" of the membrane prior to hybridization. As a result, nick-translated-DNA probe bound to the unevenly coated area forming a radioactive background.

(VI) Hybridization analysis of rat DNA intended as a control

The Southern blot analysis of rat DNA digested with Eco RI, Xba I and Xho I, and probed with v-Ha-ras oncogene are shown in figure 14. Single hybridization bands were detected in Eco RI-, Xba I-, and Xho I-digested rat DNA. They are of approximately 2.3, 2.3 and 2.0 kb. in sizes respectively.

The results are summarized in the following table 4.

Figure	Restriction enzymes	Approximate sizes of hybridization bands
14	Eco RI	2.3 kb.
	Xba I	2.3 kb.
	Xho I	2.0 kb.

Table 4.

The analysis in figure 14 were carried out in a relaxed hybridization conditions i.e. 55°C hybridization and 55°C washing.

In the course of the hybridization analysis, several different restriction endonuclease enzymes were utilized. Virtually all of the enzymes used were six nucleotide base-recognition cutters which included Hind III, Xba I, Eco RI, Pst I, Bam HI, and Xho I. There were two reasons underlying the choice of such enzymes. (1) Six nucleotide base-recognition cutters were used in order to obtain larger fragments of **Achlya** DNA. The ras-related sequences in yeast **S. cerevisiae** were found to be of 8.0 kb. and 3.8 kb. in size (DeFeo-Jones et al.,1983), therefore it was expected also that the putative ras sequence(s) in **Achlya** would be of approximately similar size. The results showed that the putative ras in **Achlya** was approximately 2.0 kb. in size. (2) Restriction endonuclease sites within the c-Ha-ras and v-Ha-ras had been mapped (Pulciani et al.,1982, Reddy,1983, Ellis et al.,1981). Based on this information, enzymes which cut outside the oncogenes or cut at one or two positons within the oncogenes were selected for use.

In the initial stage of the hybridization study, the plasmids carrying c-Ha-ras oncogene were used as probes. The use of v-Ha-ras oncogene insert as a probe, from the plasmid pHB-11, was implemented for three reasons. Firstly, since previous studies utilized v-ras as opposed to c-ras as probes (Shilo and Weinberg,1981, DeFeo-Jones et al.,1983), the absence of the intervening sequences in the v-Ha-ras might

DISCUSSION AND CONCLUSION

increase the chance of a better hybridization to the putative ras in **Achlya**. Secondly, the v-Ha-ras sequence, derived from rat genome, was evolutonarily closer to the **Achlya**. And thirdly, the pBR 322 sequence was removed to give a greater validity to the hybridization result.

Putative ras in Achlya´s genome

The hybridization analysis of **Achlya** genomic DNA indicated the presence of DNA sequences homologous to ras oncogene (see figure 9 to 13). It was demonstrated that the degree of homology between ras-related sequences in **Achlya** (putative ras) and ras oncogenes of viral or cellular origins was weak. When probings were done in a relaxed hybridization conditions (i.e. 55°C hybridization and 55°C washing) (figures 9 to 10, 12 and 13), relatively strong hybridization bands were observed. The increase of stringency by increasing the temperature of hybridization and washing conditions to 65°C, however, decreased the intensities of the hybridization bands tremendcusly. (figure 11).

The weak homology between **Achlya** putative ras and mammalian ras indicates, according to evolutionary theory, that the putative ras of **Achlya** is excessively diverged from the mammalian ones. The exact degree of homology, however, cannot be decided based on these data. Only a comparison of

the nucleotide sequences will determine the accurate degree of homology.

The smallest single hybridization band detected was approximately 2.2 kb (figure 13); therefore, the size of **Achlya** putative ras should not be longer than approximately 2.2 kb.. Nevertheless, it is also possible that the 2.2 kb. fragment is simply a portion of a longer gene where the other part has been digested to much smaller pieces that were not detected either because they were too small or bore no homology to the probe. If the putative ras exist in more than one copy, the sum of their lengths must not exceed 2.2 kb., or the multiple copies of the gene are located in a tandem array fashion with similar flanking regions.

Probable copy numbers of Achlya putative ras

The hybridization analysis of **Achlya** genomic DNA indicated that there was a single or double copies of the putative ras in **Achlya**.

The majority of the hybridization bands appeared as a single copy band. **Achlya** DNA digested with Xba I (figures 9, 10 and 11), Hind III (figures 10 and 11), Bam HI (figure 13) and Xho I (figure 13) when probed with ras oncogenes, c-Ha-ras and v-Ha-ras, exhibited single hybridization bands. Based on these data, however, the argument for the presence

of more than two copies of the putative ras cannot be dismissed. The probability for the presence of two or more copies within fragments flanked by recognition sites of the enzymes used above appears to be plausible for the large fragments of Xba I- and Hind III-digested DNA. The probability, however, is less plausible for small single fragments of Bam HI- and Xho I-digested DNA.

The two small fragments, from Hind III-digested DNA (figure 9), are probably parts of a single putative ras which contains one or two Hind III sites. This idea is consistent with the fact that there are two Hind III sites in the v-Ha-ras sequence (Ellis et al.,1981). Although there is no Hind III site within the human c-Ha-ras sequence (Pulciani et al.,1982, and Reddy,1983), it does not render the above assumption invalid, because, according to evolutionary theory, v-Ha-ras sequence derived from rat genome is more closely related to the **Achlya** than the human sequence to the **Achlya.**

The single fragments approximately 8.5 kb. in size, appearing on Hind III-digested DNA (figures 10 and 12) does not seem to represent a second copy of the putative ras in **Achlya.** In fact, the two smaller fragments found on Hind III-digested DNA (figure 9) may well be the broken-down products of the 8.5 kb. fragment. This idea is based on three facts. First, the 8.5 kb. fragment did not appear

together with the two smaller fragments. Second, the 8.5 kb. fragment (figure 10) hybridized with greater intensity than either of the smaller two fragments (figure 9) eventhough 10 µg of **Achlya** DNA was used for the analysis in figure 9 and only 5 µg of DNA was used for the analysis in figure 10. Third, the 8.5 kb. fragment appeared when the DNA was partially digested. These arguments, however, cannot exclude the probability of having two copies of the putative ras contained within the 8.5 kb. fragment.

Figure 12 shows that Pst I-digested DNA produced two hybridization bands when probed with the 2.2 kb. v-Ha-ras fragment. The v-Ha-ras sequence contains one Pst I site (Ellis et al.,1981). Human c-Ha-ras coding sequence contains four Pst I sites, but only one falls within the translated sequences (Reddy,1983). Based on these insights, there is a possibility that those two fragments hybridizing to the v-Ha-ras sequence originate from a single copy of the putative ras which contains a Pst I site.

Although the Eco RI-digested DNA produced three hybridization bands when probed with c-Ha-ras-containing plasmid (figure 9), the probability that these three fragments represent three copies of the putative ras is very unlikely for the following reasons. (1) Each hybridization band should have given a similar hybridization intensity if each represented a copy of the gene. (2) ras proteins have

molecular weights of between 20,000 to 26,000 daltons. Therefore, it is impossible for a 500 bp. gene to code for a protein of that size.

All hybridization analysis data provided the indication that there was one or two copies of **Achlya** putative ras. If the assumption that only one copy of the putative ras exists is true, it follows that this gene bears some similarities, in terms of restriction endonuclease map, with c-Ha-ras sequence from human and v-Ha-ras from rat. On the other hand, there are features which distinguish between the **Achlya** putative ras and v-Ha-ras or c-Ha-ras or both. First, there are Xba I and Xho I sites within the human c-Ha-ras sequence, and there is one Xba I site within the rat v-Ha-ras sequence. These restriction endonuclease sites appear to be missing in the **Achlya** putative ras or if they exist they must be located very close to the extreme ends of the sequence. Second, there is (are) Eco RI site(s) within the **Achlya** putative ras which is not found in either the c-Ha-ras nor the v-Ha-ras.

pBR 322 sequence interference

Several of the Southern blot analysis were performed using the whole plasmid (c-Ha-ras + pBR 322) as a probe (figures 9, 10 and 11). Therefore, there existed the possibility that it was the pBR 322 sequence rather than the

oncogene sequence which hybridized to **Achlya** genomic DNA. This argument was discarded because an independent investigator working on the Southern blot analysis of **Achlya** genomic DNA using pLMC-41 (c-myc + pBR 322) as a probe did not obtain the same or similar hybridization patterns (Singh,1986).

λ-DNA interference

The hybridization between radioactive-labelled pT24-C3 / pbC-N1 probes and λ-Hind III-digested DNA marker was observed in the analysis (figure 8). It was noticed that when such hybridization occurred, stronger hybridization always occured with the 23.1 kb., 6.6 kb. and 4.3 kb. fragments of the λ-Hind III-digested DNA marker. These hybridizations may be caused by binding of the "ori" region of the pBR 322 and a portion of λ DNA.

Hybridization between v-Ha-ras probe and rat genomic DNA intended as a control

In this analysis (figure 14), the hybridization between the v-Ha-ras probe to the rat genomic DNA produced hybridization bands at approximately 2.3 and 2.0 kb. positions. These bands had no great significance because

they were weak compared to the background smear. If they were specific hybridization bands, they could represent either the hybridizations between the ras sequences or the 30S RNA sequences.

Comparison of hybridization analysis of Achlya DNA using v-Ha-ras or c-Ha-ras as probes

Achlya putative ras showed a closer homology to human c-Ha-ras rather than rat v-Ha-ras. From this, one may conclude either that: (1) the **Achlya** putative ras is more closely homologous to the human sequence, or (2) the degree of homology is similar between **Achlya** and rat, and between **Achlya** and human sequences, but the presence of intervening sequences in the human c-Ha-ras probe actually increases the homology with the **Achlya** putative ras.

CONCLUSION

This study showed some indication that there was ras-homologous sequence(s) of DNA in **Achlya klebsiana** genome. This putative ras, however, possessed weak homology to its counterparts found in human or rat.

These findings, however, are still at the fetal stage of confirming beyond doubt the presence of ras-related sequence(s) in **Achlya**. Confirmation will only come after the sequence(s) is(are) isolated, cloned, sequenced, and the protein(s) encoded is(are) also isolated and proven to be similar to $p21^{ras}$. These should constitute further experiments.

Other aspects which may be of interest, once the ras-related gene(s) is confirmed, are: the aspect of ras-related gene(s) expression at different stages of **Achlya**´s life cycle, the function(s) of ras-related gene(s) and its protein(s), and the regulatory mechanism of the gene(s). Some related works have been performed in yeast **S. cerevisiae** (Powers et al.,1984, Tatchell et al.,1984, Kataoka et al.,1985) and in **Dictyostelium discoideum** (Reymond et al.,1984, Pawson et al.,1985).

R E F E R E N C E S

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