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Preliminary Evidence for the Possible Existence of Myc Oncogene Related Homologous Sequence(s) in *Achlya* Genomic DNA

by

© Satnam Singh

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PRELIMINARY EVIDENCE FOR THE POSSIBLE EXISTENCE OF MYC ONCOGENE RELATED HOMOLOGOUS SEQUENCE(S) IN ACHLYA GENOMIC DNA

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SATNAM SINGH

A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

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To God be the Glory

To my father and mother,

Mr. Ajaib Singh and Mrs. Amarjit K. Singh;

and to my brother Gurmit.

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ABBREVIATIONS

bp	base pair
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	kilo base pair
M.W.	molecular weight
O.D.	optical density
PVP	polyvinyl pyrrolidone
RNase	ribonuclease
rpm	revolutions per minute
Sarkosyl	N-lauroylsarcosine
SDS	sodium lauryl sulfate
TCA	trichloroacetic acid
Tris	tris (hydroxymethyl) amino methane

ABSTRACT

The Southern blots of Achlya genomic DNA cleaved with various restriction enzymes were probed with human c-myc and viral v-myc oncogene probes under non-stringent conditions (55°C). The probes detected DNA sequences homologous to v-myc and c-myc in the Achlya genome. However, the hybridization signals were relatively weak. Therefore, this indication can only be taken as preliminary evidence that there is a putative myc gene in Achlya klebsiana.

INTRODUCTION

INTRODUCTION

A variety of observations has shown that DNA of some well characterized proto-oncogenes are highly conserved within vertebrates (Stehelin et al., 1976; Collett et al., 1979; Spector et al., 1978). Recently, recombinant technology has facilitated the isolation and characterization of proto-oncogenes in invertebrate organisms such as Drosophila, yeast and Dictyostelium discoideum (Shilo and Weinberg, 1981; Pawson et al., 1985; DeFeo-Jones et al., 1983). The identification of sequences homologous to vertebrate oncogenes in invertebrates demonstrates a high degree of conservation of these genes as well as a wide distribution among divergent species.

In this thesis study, v-myc and c-myc oncogene probes were used to search for homologous sequences in the DNA of a water mould that has been designated Achlya klebsiana (LéJohn and Braithwaite, 1984). The water mould, Achlya klebsiana has been studied extensively and its life cycle has been worked out. The detection of proto-myc gene is a simple organism such as Achlya would enable one to look at its expression at different stages of the life cycle and gain new insight into its functions. Also, the spectrum of organism in which proto-oncogene structure and function could be studied would be broadened significantly. It would also provide a broader view of the evolution of oncogenes by looking for the most primitive organism in which oncogenes can be detected. The presence of oncogenes in organism very different from vertebrates would strengthen the notion that oncogenes play a role in cellular metabolism.

HISTORICAL

Animal retroviruses can be broadly divided into two categories (Duesberg et al., 1980). One is the highly oncogenic class with transforming (onc) genes and the other is the class without onc genes. In 1969, it was proposed that the transforming genes of retroviruses are present in normal cells as latent cancer genes that may be transduced by retroviruses without onc genes or activated by carcinogens (Huebner and Todaro, 1969). This view became known as the oncogene hypothesis. The paradox arising from this notion was why normal cells have evolved and maintained a class of over twenty known oncogenes which when carried by retroviruses are most potent carcinogenic agents known.

The biochemical solution of the paradox was provided by comparison of the structure of the onc genes in retroviruses with their sequence counterparts in the cellular genome (Duesberg et al., 1980; Bister and Duesberg, 1982). There are homologous counterparts of onc sequences existing in normal cells (Weiss et al., 1982; Bister and Duesberg, 1982). In rare instances, retroviruses have transduced these sequences from cells to generate viral onc genes.

The biochemical definition of onc genes provided the basis for testing their postulated cellular origin. The search for oncogenes in cellular DNA began with molecular hybridization of cDNA probes made from Rous sarcoma virus RNA to cellular genome (Stehelin et al., 1976; Spector et al., 1978). Many vertebrates including fish, birds and mammals displayed evidence of both DNA and RNA related to the src oncogene (Spector et al., 1978). These findings led to the following suggestions. First, all vertebrates possess a highly conserved gene

(c-src) that is related to viral src and is potentially oncogenic. Second, viral src oncogene arose by transduction of cellular src into pre-existent retrovirus (Bishop, 1983). The subsequent findings of cellular prototypes of the ras gene of Kirsten and Harvey sarcoma virus (Scolnick et al., 1973; Scolnick et al., 1974; Tsuchida et al., 1974) provided systems for biochemical analyses of oncogenes.

As more retroviral oncogenes became known, each in turn proved to have a cellular homologue from which the viral gene was apparently derived (Weiss et al., 1982; Bishop, 1981). Homologues of about 20 viral onc genes have been found in normal cells (Weiss et al., 1982). A current listing of the various associated cellular oncogenes is shown in Table I.

The retrovirologists have adopted the nomenclature where viral oncogenes are termed v-oncs and the cellular homologues of the viral oncogenes being known as cellular oncogenes (c-oncs). The names of each viral and cellular oncogenes are deduced from the virus in question. For example, v-src, c-src, v-myc, c-myc. Until recently, proto-oncogene was the term used to define the cellular homologues of retrovirus v-oncs (Bishop, 1981) as well as the cellular genes whose damage leads to active oncogenes in turmour DNA (Weinberg, 1982a; Weinberg, 1982b). Recently, Bishop (1983) has defined proto-oncogene as any cellular gene that has the potential to become an oncogene whereas the term cellular oncogene has been preserved for active oncogenes in tumour DNA.

The extent of evolutionary conservation of cellular oncogenes varies from one oncogene to another. The evolutionary age of oncogenes is based on the assumption that conserved function demands preservation of nucleotide sequence. Some cellular oncogenes are found only in

closely related species and therefore it is difficult to date them whereas some oncogenes can be dated prior to the emergence of vertebrates (Shilo and Weinberg, 1981). The evolutionary conservation of cellular homologues of viral oncogenes implies essential and fundamental roles for these genes in normal cellular metabolism (Gallwitz et al., 1983; DeFeo et al., 1983).

Structural Characteristics of Cellular Oncogenes

The advent of molecular cloning has facilitated the isolation and characterization of homologous c-oncs from organisms such as humans, Drosophila and yeast (Watson et al., 1983; Shilo and Weinberg, 1981; DeFeo-Jones et al., 1983). The cellular prototypes of most known viral onc genes have been cloned and compared with viral counterparts by heteroduplex analysis, restriction endonuclease site mapping and complete sequence analysis (Oskarrsson et al., 1980). The studies have shown that c-onc genes are cellular genes and not viral genes in disguise. Although few of the isolated genes have been characterized in detail, it nevertheless shows that homologous c-oncs transduced from different species share common structural features. First, the c-onc genes behave as classical Mendelian loci. The location of c-onc genes is at constant genetic loci in every member of a species (Hughes et al., 1979; Hughes et al., 1980). Second, there are intervening sequences within many of the c-onc genes (Groff et al., 1980; DeFeo et al., 1981; Franchini et al., 1981; Parker et al., 1981; Shalloway et al., 1981; Takeya et al., 1981).

Mechanisms of Activation of Proto-oncogenes

There are basically five separate mechanisms of proto-oncogene

activation that have been discovered so far. The first mechanism involves overexpression of a proto-oncogene after it acquires a transcriptional promoter. The mos proto-oncogene of mice, which is biologically inactive after molecular cloning, can be converted experimentally into a potent oncogene by addition of a strong transcriptional promoter (Blair et al., 1981). Similarly, Ha-ras proto-oncogene of rats was also activated by addition of a strong transcriptional promoter (DeFeo-Jones et al., 1981). The myc and erb B proto-oncogenes are present in several avian hematopoietic neoplasias. They have been shown to become activated by adjacent integration of an avian leukosis proviral DNA segment. The viral segment has a strong transcriptional promoter that overrides the promoters of these genes (Hayward et al., 1981; Payne et al., 1982; Fung et al., 1983).

The second mechanism is gene amplification. In several cases, a proto-oncogene has been found to be present in large numbers of copies per cell instead of the normal two copies characteristic of most cellular genes. This amplification of the gene involves the proto-oncogene being somehow replicated until it is present in a cell in multiple copies, either sequentially repeated segments along the chromosome or dissociated extrachromosomal particles resulting in inappropriate overexpression (Weinberg et al., 1983). The myc proto-oncogene is amplified 30 to 50 times in the human promyelocytic leukemia cell line HL-60 (Collins and Groudine, 1982; Dalla-Favera, 1982). A Ki-ras gene is amplified three to five times in a human colon carcinoma cell line (McCoy et al., 1983). The human chronic myelogenous leukemia cell line was found to have extra copies of the cellular abl gene (Collins and Groudine, 1983). In all the above mentioned cases,

the increased copy number is presumed to cause corresponding increases in transcript and gene product.

The third mechanism involves the activation of proto-oncogenes by the action of "enhancer" sequences which can influence the level of transcription and hence the amount of the gene product. The mechanism of action of enhancer sequences is not fully understood but they seem to increase the utilization of transcriptional promoters to which they become linked. It has been found that the linked promoter may be several kilobases away and the enhancer sequence may be positioned upstream or downstream of the promoter (Gruss et al., 1981; Levinson et al., 1982). One such enhancer is retrovirus genome fragment that is downstream from the myc gene is avian lymphomas (Payne et al., 1982).

A fourth mechanism is chromosomal rearrangement whereby an hitherto silent or tightly regulated proto-oncogene is activated. This mechanism relates to the activation of myc proto-oncogene whose actively oncogenic version has been found in mouse plasma cytomas and human Burkitt's lymphomas (Leder, 1983). The cellular proto-oncogene becomes separated from its usual position at the end of one chromosome and is translocated to the end of a second chromosome. There it becomes juxtaposed with genes responsible for the synthesis of immunoglobulins. This appears to result in deregulation of the myc gene, which loses regulatory sequences of its own and acquires instead sequences involved in immunoglobulin production (Leder, 1983) and this causes proto-oncogene's genetic information to be expressed at an inappropriate level. Recently, rearranged myb sequences have been detected in mouse plasmacytomas (Mushinski et al., 1983).

The fifth mechanism involves point mutation of the proto-oncogene

in a normal cell by radiation or a chemical carcinogen. The mutation may change the information encoding a protein. This mechanism is best understood in the case of the oncogene proteins encoded by ras genes. It was found that a point mutation, in the case of the human bladder carcinoma, converted the Ha-ras proto-oncogene into a potent oncogene. The G to T transversion caused the glycine normally present as the 12th residue of the 21,000 M.W. protein to be replaced by valine (Capon et al., 1983). It seems that this change does not affect the level of expression of these genes, only the structure of the encoded proteins.

Proteins Encoded by Oncogenes

It has been difficult to unfold the biochemical functions of oncogenes. The pertinent question that arose was what are the proteins encoded by oncogenes and how do they transform cells? Whatever may be the mechanism by which a proto-oncogene is converted into an oncogene, the oncogene must finally have its effect in the protein it encodes (Hunter, 1984). It has been difficult to characterize the proteins encoded by cellular oncogenes because of their availability in small amounts.

In 1978, Collett and Erikson isolated the product of c-src from RSV (Rous sarcoma virus) infected cells and found that it is a 60,000 dalton phosphoprotein ($pp60^{c-src}$). It was discovered that $pp60^{c-src}$ had a tyrosine specific protein kinase activity that was remarkably similar to that of the protein encoded by v-src ($pp60^{v-src}$) (Collett et al., 1979b; Levinson et al., 1978; Collett et al., 1979; Oppermann et al., 1979; Rohrschneider et al., 1979; Collett et al., 1980; Hunter and Sefton, 1980; Levinson et al., 1980; Sefton, 1980; Karess and Hanafusa,

1981). The proteins of other known oncogenes have also been tested for tyrosine specific protein kinase activity. The products of yes, fgr, abl, fps, fes and ros oncogenes have been shown to have tyrosine specific protein kinase activity (Hunter, 1984).

In light of this finding, it became important to know how the addition of phosphate to tyrosine in a cell's protein cause the varied effects of transformation? The understanding of this lay in the identification of proteins that are targets for protein kinases. The cytoskeleton of the cell is altered in cells transformed by a tumour virus. Therefore, the cytoskeleton proteins were looked at as potential targets of tyrosine specific protein kinases. Of the 10 cytoskeletal proteins isolated from RSV transformed cells, only the protein vinculin was found to contain phosphotyrosine. Vinculin is localized in adhesion plaques which are specialized regions of the plasma membrane that account for the adherence of cultured cells to glass or plastic. The vinculin may connect the actin filaments to anchor protein in the membrane and the increased phosphorylation of the tyrosines in vinculin may reduce its tenacity as a linker and lead to the release of actin filaments (Hunter, 1984). Another potential target protein has been identified for the enzymatic activity of $pp60^{c-src}$ in vivo (Radke and Martin, 1979). It is a cellular protein with a molecular weight of 36,000 (p36) (Radke and Martin, 1979; Erikson and Erikson, 1980; Radke et al., 1980; Purchio et al., 1981). The function of p36 in the cell is not known, although it may be attached to components of the cytoskeleton (Cooper and Hunter, 1982). The phosphorylation of tyrosine has been implicated in the cellular response to mitogenic growth factors like epidermal growth factor (EGF) and the platelet derived growth

factor (PDGF) (Ek et al., 1982; Nishimura et al., 1982; Kasuga et al., 1982; Cooper et al., 1983). This means that the signal to divide could be transmitted from the occupied receptor to the interior of the cell by phosphorylation on tryosine of target proteins in the cell (Ushiro and Cohen, 1980; Kasuga et al., 1982). The enzymatic activity of $pp60^{c-src}$ suggests that it may be involved in the control of cell growth and division.

The two families of c-ras genes encode for similar proteins with a molecular weight of 21,000 ($p21^{c-ras}$) (Langbeheim et al., 1980; Furth et al., 1982). The ras encoded proteins bind to guanosine triphosphate (GTP) forming a stable complex (Shih et al., 1980; Scolnick et al., 1981). The protein has a function homologous to the G protein and together with GTP binds to effector adenylate cyclase (Hunter, 1984).

There is also a small group of nuclear transforming proteins. It includes the products of myc, myb and fos oncogenes (Donner et al., 1982; Abrams et al., 1982).

There is relatively little known about other cellular oncogene products. The protein encoded by c-fps has a molecular weight of 98,000 ($p98^{c-fps}$) and displays tyrosine specific protein kinase activity like that of the viral protein (Mathey-Prevot, et al., 1982). The product of c-abl is 150,000 molecular weight protein ($p150^{c-abl}$). There is nothing known about its function other than that tyrosine specific kinase activity like that of the v-abl protein has not been found (Ponticelli et al., 1982).

The v-myc oncogene is homologous to a cellular gene (c-myc) that is present in normal uninfected chicken DNA (Sheiness et al., 1980; Sheiness and Bishop, 1979) and has been shown to be highly conserved throughout evolution in organisms as diverse as man, chicken and Drosophila (Robins et al., 1982; Sheiness and Bishop, 1979; Shilo and Weinberg, 1981; Vennstrom et al., 1982; Dalla-Favera et al., 1982).

v-myc Oncogene Homologues in Vertebrates

Sheiness and Bishop (1979) observed that the genomes of several birds and other vertebrates included a highly conserved homolog of cDNA to v-myc. The homology was detected by hybridization of $cDNA_{MC29}$ (cDNA to v-myc) to DNA and RNA of various avian species. The DNA complementary to the putative transforming gene of avian myelocytomatosis virus MC29 annealed under stringent conditions (0.6 M Nacl, 68°C) to DNA from normal chickens as well as to DNA from several birds phylogenetically diverged from chickens. The hybridization of $cDNA_{MC29}$ was virtually complete in the case of the chicken genome with 90% of the $cDNA_{MC29}$ annealing to RNA. When the duplexes consisting of $cDNA_{MC29}$ and RNA were thermally denatured, it did not reveal any mismatched base pairs.

Dalla-Favera et al. (1982) studied the genomic organization of human cellular sequences (c-myc) homologous to the transforming gene (v-myc) of avian myelocytomatosis virus. They used v-myc probes to hybridize to Southern blots of human genome. They observed that several fragments of the human genome contain sequences homologous to v-myc. Several recombinant phages were isolated from a human DNA library, representing regions of the genome containing c-myc related sequences.

Two clones were found to overlap approximately 17 kilobases of DNA where a sequence homologous to that of the entire v-myc was present. Three different clones containing human c-myc sequences were also isolated and found to be homologous to the central portion of the v-myc gene and had no intervening sequence. Dalla-Favera et al. (1982) suggest that these sequences may represent different parts of different functional genes which are partially homologous with the c-myc gene or they may not be functional and may represent pseudogenes.

The ubiquitous distribution of sequences homologous to v-myc gene in vertebrate genomess suggested that these sequences may encode some vital cellular function. First, the c-myc sequence is highly conserved throughout vertebrate species. Second, the c-myc gene is transcribed into polyadenylated RNA. Third, the size of the RNA (2.5 Kb) is constant among several avian and mammalian species, in the manner of mRNAs for conserved proteins such as globin and lens cystallin (Taylor, 1979).

v-myc Homologues in Invertebrates

In order to get a broader view of the evolution of proto-oncogenes by looking for the most primitive organism in which these genes could be detected, retrovirus derived oncogene sequences were used as probes to search for homologous sequences in the DNA of Drosophila melanogaster (Shilo and Weinberg, 1981).

Drosophila melanogaster DNA was cleaved with various restriction enzymes prior to electrophoresis and gel filter transfer by Southern blot procedure (Southern, 1975). The plasmid carrying v-myc gene was labeled by nick translation (Rigby et al., 1977) and hybridized to

nitrocellulose filters carrying DNA from Drosophila melanogaster. The probe hybridized with multiple homologous sequences in Drosophila DNA. The multiplicity of bands could not be accounted for solely by cleavage of a single stretch of homologous sequence into many DNA fragments. The probe seemed to have detected a family of genes in the Drosophila genome.

The ability to detect sequences homologous to vertebrates oncogenes in invertebrates demonstrated a high degree of conservation of these genes. Shilo and Weinberg (1981) estimated that the common precursors of these genes were already evolved 800 million to a billion years ago and should be conserved in the organisms of the Echinoderm-chordate and the Annelid-Arthropod Superphyla.

Therefore, the identification of sequences homologous to vertebrate oncogenes in invertebrates demonstrated a high degree of conservation of these genes as well as suggesting a role for them in cellular metabolism.

Structural Characteristics of the v-myc and c-myc Oncogenes

The avian myelocytomatosis virus strain MC29 is a replication defective acute leukemia virus (Dalla-Favera et al., 1982) capable of transforming fibroblasts and macrophage like cells in vitro (Beug et al., 1973; Graf, 1973). It includes a wide spectrum of tumours including carcinomas, endotheliomas and sarcomas (Graf and Beug, 1978; Beard, 1980; Moscovici, 1978). The oncogenic potential of the retrovirus is mediated by the gene v-myc which is located approximately in the middle of the 5.5 kilobase viral genome. The v-myc gene consists of 1,568 base pairs (Duesberg et al., 1977; Hu et al., 1979; Sheiness

et al., 1980) that may encode the oncogenic capacity of the virus (Duesberg et al., 1978). The virus contains no other functional genes since only portions of the gag and env genes are present and pol is deleted entirely (Duesberg et al., 1977; Hu et al., 1979). As a result, the virus is replication defective and requires a helper virus to propagate. The v-myc gene is fused to a portion of the gag gene which encodes the internal structural proteins of the virus. This hybrid gene gives rise to a polyprotein with a molecular weight of 110,000 ($p110^{gag-myc}$) (Bister et al., 1977).

The oncogene v-myc is homologous to a cellular gene (c-myc) that is present in several avian and mammalian species (Roussel et al., 1979; Sheiness and Bishop, 1979). The c-myc gene consists of three exons. The first exon encodes two transcription initiation points. The translational initiation codon is in exon 2 while the translational termination codon is at the end of exon 3. There is a polyadenylate site after exon 3 (Saito et al., 1983). The alignment of the chicken c-myc sequences with that of v-myc reveals that the intervening sequences in the c-myc can be identified by consensus splice signals. The homology between v-myc and c-myc is conserved in the coding regions or exons 1 and 2 of c-myc; and most changes at the nucleotide level result in no changes in the amino acid (Watson et al., 1983b). The 5' domain of c-myc is unique and is different from the viral 5' domain. However, the variation between v-myc and chicken c-myc is less than 2% at the 3' domain (Watson et al., 1983a).

Protein of myc Oncogene

The putative transforming protein of avian myelocytomatosis virus

MC29 is a 110,000 dalton protein ($p110^{gag-myc}$) (Hu et al., 1979; Mellon et al., 1978). By reference to the physical map of MC29 genome, it was deduced that $p110^{gag-myc}$ represented a fusion between gag and v-myc (Rettenmeier et al., 1979). It has been estimated that the removal of the gag region of the 110,000 dalton protein would yield a v-myc specific protein with a molecular weight of about 55,000 daltons. The c-myc oncogene is not associated with any gag like DNA sequences and could not encode a homolog of the 110,000 dalton protein. However, the 2.8 Kb transcript of c-myc has the ability to code for a 55,000 dalton protein that corresponds to the hypothetical cleavage product of $p110^{gag-myc}$ (Sheiness et al., 1980).

The $p110^{gag-myc}$ protein is phosphorylated on serine within the gag region and in both serine and threonine within the myc region (Hunter et al., 1981). The $p110^{gag-myc}$ protein shows no kinase activity and binds to double stranded DNA with high affinity (Donner et al., 1982).

Recently, Watt et al. (1985) have demonstrated the expression of the modified human c-myc gene in E. coli. The myc protein has an apparent molecular weight of 64,000 and is not phosphorylated like the myc product in eucaryotic cells. However, upon microinjection of purified protein into frog oocytes, the protein is phosphorylated. Watt et al. (1985) are currently investigating the nature of this phosphorylation. The c-myc protein expressed in E. coli retains its DNA binding activity.

The ability to express c-myc protein in E. coli will provide enough protein to enable detailed study of the biochemical characteristic of this oncogene product.

Nuclear Association of myc Oncogene Protein

Abrams et al. (1982) demonstrated a nuclear association of the putative transforming protein of MC29. The approach employed anti-gag sera to detect MC29 gag related polyproteins. The MC29 protein $p110^{gag\text{-}myc}$ and a minor variant $p100^{gag\text{-}myc}$ appeared to be localized in the nuclear fractions of MC29 transformed quail embryo fibroblast cell line, MC29-Q8-NP, which does not produce virus particles but expresses the transforming protein gag-myc.

The purity of the nuclear fraction was assessed by electron microscopy and biochemical analyses. The results of enzyme assays and electron microscope studies indicate that nuclear localization of $p110^{gag\text{-}myc}$ in MC29 transformed cells is not due to non-specific binding of cytoplasmic and membrane contaminants.

Indirect immunofluorescence studies using multispecific and monospecific anti-gag sera demonstrated intense and specific fluorescence within the nuclei of MC29-Q8-NP cells. The staining pattern observed suggested that $p110^{gag\text{-}myc}$ isolated mainly within the nucleus and is not associated with the nucleoli or the nuclear envelope. The protein has been reported to have a DNA binding capacity (Donner et al., 1982).

Regulation of c-myc Oncogene

Current knowledge of cellular growth control allows one to make predictions regarding proto-oncogene expression if, in fact, they are involved in regulation of normal growth. Recent observations seem to link certain oncogene products to different compartments along the mitogenic pathway of the normal cell. Kelly et al. (1983) have shown

that c-myc is an inducible gene that is regulated by specific growth signals in a cell cycle dependent manner. The purpose of their experiments was to establish where the product of the c-myc gene might act in the successive steps by which cells progress through their growth cycle.

The induction of c-myc mRNA is regulated by agents that stimulate a strong growth response in lymphocytes (concanavalin A and lipopolysaccharide) and fibroblast [platelet derived growth factor (PDGF)]. The growth of fibroblasts (Pledger et al., 1977) and lymphocytes (Baserga, 1983) can be divided into roughly three phases, each triggered by different tissue specific growth factors. The first phase is known as the phase of priming for growth competence, and it is in this phase that c-myc is implicated. The induction of competence is accompanied by cellular enlargement (DeFranco et al., 1982), increase in RNA content (Zbigniew et al., 1979) and the synthesis of noval protein species (Pledger et al., 1981; Milner and Milner, 1981).

Kelly et al. (1983) found that the addition of the mitogens concanavalin A, lipopolysaccharide and PDGF to the appropriate cells stimulates the transient expression of c-myc within one to three hours. There is a 10 to 40 fold increase in c-myc mRNA concentration during this period and it returns to baseline well before the onset of DNA synthesis. This induction of c-myc mRNA was shown to occur in presence of cycloheximide. Therefore, it implies that synthesis of new protein is not required and the induction of c-myc mRNA cannot be a secondary effect of growth. In addition, there was superinduction of c-myc mRNA following cycloheximide and mitogen treatment of cells. This result is consistent with the idea that a labile protein may regulate the level of

c-myc mRNA (Leder, 1983).

Co-operation of ras and myc Oncogenes

The conversion of normal cell to a malignant one seems to be a multifactorial process. Recent experiments support the model of multihit character of carcinogenesis (Land et al., 1983; Ruley, 1983; Newbold and Overell, 1983).

Land et al. (1983) described a transfection assay that is dependent on c-myc activity. They found that co-transfection of viral promoter linked c-myc genes and mutant ras genes could transform secondary rat embryo fibroblasts. In conditions in which either ras or myc had no obvious effect on the monolayer cultures, the two genes together achieved alteration of phenotype. The foci carrying ras and myc genes yielded rapidly growing cultures of morphologically transformed cells. These co-transfected cells were tumorigenic when introduced into nude mice yielding tumours.

It has also been found that two different viral early genes, adenovirus E1A (Ruley, 1983) and polyoma large T (Land et al., 1983) can substitute for c-myc in the assay, thereby implying there is some overlap among the proteins. A limited amount of protein sequence homology has been identified between c-myc and E1A (Ralston and Bishop, 1984).

Activation of c-myc Oncogene

Activated or amplified forms of c-myc oncogene have been found in a wide range of tumours, including small cell lung carcinomas (Little et al., 1983), murine retrovirus induced thymomas (Steffen, 1984; Corcoran et al., 1984), a promyelocytic leukemia (Collins and Groudine, 1982;

Dalla-Favera et al., 1982), and a neuroendocrine tumour (Alitalo et al., 1983). The avian leukosis virus, which lacks an oncogene and which induces bursal lymphomas, integrates next to c-myc and increases the expression of c-myc (Hayward et al., 1981; Payne et al., 1981). More recently, it has been observed that activation of c-myc in some Burkitt lymphomas may be accomplished via point mutations within the gene or in the regulatory regions (Rabbitts et al., 1983). Even though the mutations may play a significant role, their extent is variable (Taub et al., 1984).

In murine plasmacytomas (Shen-Ong et al., 1982; Adams et al., 1982; Marcu et al., 1983) and in Burkitts lymphomas (Taub et al., 1982; Erikson et al., 1983), the myc gene is perturbed by translocations of this oncogene into one of the immunoglobulin loci (Leder, 1983). These translocations occur at many different positions within and near myc and since myc is joined to different regions of the immunoglobulin loci, sometimes juxtaposing the immunoglobulin enhancer with myc (Taub et al., 1983; Bernard et al., 1983; Cory et al., 1983; Dalla-Favera et al., 1983; Hollis et al., 1984). This generally results in an increase in myc transcription, although it is variable (Westin et al., 1983; Hamlyn and Rabbitts, 1983; Maguire et al., 1983; Taub et al., 1984).

In almost all instances, the c-myc protein coding regions are unaltered (Battey et al., 1983) indicating that the same protein is produced from the activated and normal genes. However, it has been frequently observed that the first exon (untranslatable) and/or 5'-flanking sequences of the c-myc gene are displaced. Furthermore, both in plasmacytomas (Adams et al., 1983) and in Burkitt lymphomas (Nishikura et al., 1983; Leder, 1983; Taub et al., 1984), the

germ-line allele is inactive while the translocated allele is transcriptionally active.

All these observations have prompted several models that attempt to explain the activation of c-myc gene. The primary models proposed include: 1) Increased translational efficiency of altered c-myc mRNA (Saito et al., 1983); 2) Deregulation of c-myc expression due to displacement of a repressor (Leder et al., 1983).

As stated previously, the loss of exon 1 may be a common feature of most human Burkitt lymphomas and murine plasmacytomas. The examination of the c-myc sequence reveals that there is a region of high complementarity between exon 1 (nucleotides 283-350) and exon 2 (nucleotides 2456-2526) (Saito et al., 1983). Saito et al. (1983) has proposed a stem loop secondary structure for the human c-myc RNA. The initiator AUG codon would be located within the loop. The 40S ribosome subunit, according to "bind and scan" model for eukaryotic translation, binds the 5'-terminus of mRNA and migrates toward the initiator AUG codon (Kozak, 1978). When the secondary stem loop structure is introduced into c-myc mRNA, the binding and migration of the 40S ribosome subunit is hindered. Therefore, the initiator AUG codon would be inaccessible to initiation of translation in the secondary stem loop structure. Whereas, in the c-myc RNA from the translocated c-myc gene which lacks exon 1, a stem loop structure cannot be generated. Therefore, the transcript from the translocated c-myc gene can be translated at a much higher efficiency than the transcript from the non-translocated c-myc gene. This model, however, cannot be applied to explain the cases in which c-myc rearrangements occur at some distance from the c-myc gene (Adams et al., 1983). Saito et al. (1983) suggest

that the overproduction of the c-myc in these cases may be due to transcriptional effects (Mushinski et al., 1983; Maguire et al., 1983). The rearranged c-myc gene is brought close to sequences that have tissue specific transcription enhancing activity (Gillies et al., 1983; Banerji et al., 1983).

On the other hand, Leder (1983) and his colleagues have put forward another model to explain all these observations in Burkitt lymphomas. Leder et al. (1983) state that the 5'-untranslated region including exon 1 is the binding site for a transcriptional repressor which may be induced by or identical to the c-myc product itself. In most Burkitt lymphomas and murine plasmacytomas, exon 1 of c-myc is either lost or truncated during translocation. The lack of binding site for a transcriptional repressor in the translocated gene results in increased expression of c-myc gene. However, this model does not comply with the case where there is constitutive expression of c-myc after the c-myc gene is translocated intact. This constitutive expression could be induced by currently undefined enhancing elements within the Ig domain (Keath et al., 1984).

As one can see, there clearly exists multiple mechanisms by which c-myc gene can be activated.

TABLE 1

NAME OF ONCOGENE			ONCOGENIC PROTEIN		
	RETROVIRUS	TUMOR	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	—	(?)	(?)	

The table of oncogenes, the retroviruses and tumors associated with them, their cellular location and the oncogenic proteins encoded by them (Hunter, 1983).

MATERIALS AND METHODS

I Organism

Achlya: Achyla species (Class, oomycetes; order saprolegniales; and family, saprolegniaceae (Alexopoulos, 1962; Dick, 1973).

The water mould used in this thesis study has been designated Achyla klebsiana (LeJohn, 1983) and was obtained from Dr. J. S. Lovett, Purdue University, U.S.A. Achlya klebsiana is found in water and soil (Webster et al., 1970) and the stages of its life cycle have been examined and described by Cameron and LeJohn (1972).

II Preparation of Cultures

Growth media: During the course of this study, the following media were most commonly used.

(a) G_2Y Medium:

Glucose	5.0	g
Yeast extract (Difco)	0.5	g

Dissolve in 1 liter of tap distilled water.

(b) Defined Medium:

The procedure described by Barksdale (1963) was used to prepare this medium.

Monosodium glutamate	0.5	g
Glucose	2.8	g
L-cystine*	1	ml
KH_2PO_4	1.5	mM
KCl	2.0	mM
$MgSO_4 \cdot 7H_2O$	0.5	mM
$CaCl_2$	0.5	mM
Na_2EDTA	.01	g
Tris	1.2	g
Metal mix (#4 solution)	10.0	ml

. . . . . to 1 liter of tap distilled water.

*1 ml of 20 mg/ml HCl (0.1N) solution.

The pH was adjusted to 6.8 with 1N HCl prior to the

addition of $CaCl_2$.

A 100 ml of metal mix (#4 solution) was prepared by mixing 100 mg sulfosalicylic acid and 200 mg of a ground mixture of Fe $(NH_4)_2 (SO_4)_2 \cdot 6H_2O$ (28.9 g), $ZnSO_4 \cdot 7H_2O$ (8.8 g), and $MgSO_4 \cdot H_2O$ (3.1 g). Glucose was autoclaved separately and added prior to use of the medium.

III Growth of Organism

(a) Stock Cultures

Achlya cultures were maintained in plastic petri plates containing 20 ml of G_2Y medium. In G_2Y medium, sporulation occurs within 48 hours at room temperature. After a week, the mycelial mats were stiff enough for lifting out of the plates. Five mycelial mats were transferred to autoclaved plastic bottle containing 100 ml sterile tap distilled water. The bottle was shaken vigorously for 30 seconds to release the spores from the mat. The mats were removed from the spores by filtration through nylon mesh (66 µm pore size) and used as inoculum. The inoculum consisted of 2 ml of spore suspension in 20 ml of G_2Y medium. This procedure was repeated every week to maintain a stock supply of standardized inoculum.

(b) Culture in Defined Medium

Whenever large amount of spores were required for the purpose of isolation of DNA, Achlya hyphal mats were grown in 20 ml of defined medium supplemented with 0.5 mM glutamine in plastic petri dishes. In defined medium, hyphal mats would show good sporulation within 4 - 5 days at room temperature.

The mycelial mats from the G_2Y medium were transferred to sterile plastic bottle containing 100 ml tap distilled water. The bottle was shaken vigorously for a minute to release the spores from the mat. The mats were removed from the spores by filtration through nylon mesh and used as inoculum in defined medium. The inoculum consisted of 2 ml of spore suspension in 20 ml of defined medium.

(c) Large Scale Preparation of Germinated Spores (germlings)

The spores were collected by shaking the mat along with the spent medium in a large sterile plastic screw capped bottle. The suspension of cells was filtered through nylon mesh so that only spores were recovered in the filtrate. The spore suspension was used as inoculum for sterile G_2Y medium. Two hundred milliliter of spore suspension was used for every 500 ml of G_2Y medium. The cells were chilled overnight at 0°C. The flasks with G_2Y medium and the spores were immersed in ice baths and left in the cold room overnight. Following chilling, the flasks were transferred to a large floor model reciprocating shaker at 28°C. The cells were agitated vigorously at 160 rev/min for 3 hours. Germlings were recovered by filtration.

IV Isolation of DNA

(a) Solutions and Buffers

(1) Bacterial Protease

The stock solution of the enzyme at 100 mg/ml concentration was made in 50 mM Tris.Cl, pH 8. The

solution was autodigested at 37°C for 1 h. The solution was aliquoted in Eppendorf centrifuge tubes and stored at -20°C.

(2) Ribonuclease (DNase free)

Ribonuclease (RNase A) was dissolved at a concentration of 10 mg/ml in 10 mM. Tris.Cl (pH 7.5) and 15 mM NaCl. The solution was heated to 100°C for 15 minutes and allowed to cool slowly to room temperature. It was dispensed into aliquots and stored at -20°C.

(3) Buffer A

1% Dresilase in 0.5 M $MgSO_4$, 0.05 M K-maleate, pH 5.8. This buffer was clarified by filtering through Nalgene filter (0.45 μm) prior to use.

(4) Buffer B

0.5 M $MgSO_4$, 0.05 M K-maleate, pH 5.8.

(5) Lysis Buffer

1% Sarkosyl in 0.1 M NaCl, 0.1 M Na_2EDTA, 50 mM Tris.Cl, pH 8.0.

(6) Ethidium Bromide (EtBr)

20 mg/ml

(7) CsCl

Used as crystals.

(8) Tris.EDTA (TE) Buffer (per liter)

10 ml of 1 M Tris-Acetate, pH 7.5

4 ml of 0.25 M Na_2EDTA

986 ml of Deionized water

(9) CsCl Saturated Isopropanol

100 to 200 millilitres of isopropanol in storage bottle; CsCl crystals added in small batches with stirring, until no more dissolved.

(b) Method

The following method was used with 20 plates of Achlya grown in defined medium. The germlings were collected by filtering through 10 µm nylon millipore filter (SM). The flask was washed with 50 ml of tap distilled water. The germling mat was suspended in 20 ml of buffer A and incubated in 37°C water bath for approximately 1 h with gentle shaking. A drop of the cell suspension was examined microscopically before and after sphaeroplasting. The sphaeroplasts that had formed were uniformly round in contrast to the flask-shaped germlings. A drop of the sphaeroplast was added to a drop of 2% sarkosyl detergent. This was incubated on ice for 5 min and then examined under the microscope. The cells lyzed if sphaeroplasts had formed. If not, the cells were incubated in sphaeroplasting solution for a further 30 min and the test repeated.

When sphaeroplasting was complete, the sphaeroplasts were centrifuged for 5 min at 2000 x g at 4°C. The supernatant was decanted carefully and the cells were resuspended in 10 ml of Buffer B and recentrifuged for 5 min at 2000 x g. The supernatant was decanted and the pellet was dissolved in 8 ml of lysis buffer and stirred gently.

RNase (DNase free) was added to the solution at a

concentration of 100 µg/ml. It was incubated in a 37°C water bath for at least 3 h and sometimes overnight. Protease (0.1 mg/ml) was added to the lysed sphaeroplasts and incubated for 3 h. Following protease digestion, the lysed protoplast solution was poured into a beaker containing 8 g CsCl. The beaker was swirled to dissolve the CsCl. When CsCl was completely dissolved, 0.2 ml of the ethidium bromide (20 mg/ml) solution was added. The protoplast lysate was put into a quick seal Beckman ultracentrifuge tube (5/8 x 3 in) and filled with paraffin oil. The tubes were centrifuged in a Beckman ultracentrifuge (L8-80) in a 80Ti rotor at 60 K for 20 h at 14°C. The tubes were examined through vertically projected U.V. light. The plug was removed from cap before puncturing the tube from the side with a hypodermic needle (20 Gl) just below the DNA band. The DNA band was collected in 1 ml solution by hypodermic and put in a small but wide mouth test tube. One millilitre of CsCl-saturated isopropanol was added and the test tube was shaken gently for about 1 min. Then, the phases were allowed to separate. Using a pasteur pipette, the top phase was discarded. The procedure was repeated until colour was practically gone from the DNA aqueous phase.

The DNA solution was transferred to double knotted dialysis bag and dialysed against Tris-EDTA (TE) buffer (500 ml) overnight. The buffer was changed the next day and dialysis was continued for another day. The DNA was then transferred to siliconized Eppendorf tubes and stored in the refrigerator. A drop of chloroform was added to avoid microbial

contamination.

The concentration of the DNA was determined in Gilford 2400 spectrophotometer based on the rule that 1 O.D. at 260 nm is equal to 50 µg/ml of DNA.

V Large Scale Isolation of E. coli Plasmid

(a) Media Required for Plasmid Isolation

LB (Luria Broth)

Bacto typtone	10 g
Bacto yeast extract	5 g
NaCl	5 g

. . . . . add to 1 litre of tap distilled H_2O.

Dissolve and adjust pH to 7.

(b) Solutions Required for Plasmid Isolation

(1) Sucrose Buffer

15% sucrose
50 mM Tris-Acetate, pH 8
50 mM Na_2EDTA, pH 8

(2) Triton Buffer

50 mM Tris-Acetate, pH 8
50 mM Na_2EDTA, pH 8
0.4% Triton x 100

(3) Tris-EDTA (TE) Buffer

10 ml of 1 M Tris-Acetate, pH 7.5
4 ml of 0.25 M Na_2EDTA
986 ml of Deionized water

(4) Ethidium Bromide (EtBr) solution

20 mg/ml

(5) CsCl

Used as crystals.

(6) CsCl-Saturated Isopropanol

100 to 200 millilitres of isopropanol in storage bottles; CsCl crystals added in small batches with stirring until no more dissolved. As it became depleted, it was replenished by adding more isopropanol.

(7) Chloramphenicol

34 mg/ml in ethanol

(c) Method

(1) Plasmid Amplification with Chloramphenicol

Screw capped test tubes containing 10 ml LB medium with the appropriate antibiotic were inoculated with a loopful of bacteria. The test tubes were incubated at 37°C overnight with shaking. The following morning, 10 ml of the late log phase culture was inoculated into 500 ml LB medium prewarmed to 37°C with the appropriate antibiotic. It was then incubated for approximately 8 h at 37°C with vigorous shaking (OD_{600} of the culture was approximately 0.4). Two and a half millilitres of the solution of chloramphenicol (34 mg/ml in ethanol) was added. The final concentration of the chloramphenicol in the culture was 170 µg/ml. It was incubated at 37°C with virgorous shaking for a further 12 - 16 h. The cells were harvested and large scale isolation of plasmid was carried out.

(2) Large Scale Isolation of E. coli Plasmid

The cells (1 litre) from overnight culture were harvested and spun down in 500 ml plastic centrifuge

bottles at 8,000 x g for 10 min at 4°C. The cell pellet was resuspended in 2 - 3 ml of sucrose buffer using a rubber policeman and pasteur pipette. It was kept on ice throughout. The cell slurry was transferred to screw capped small centrifuge tubes. About 30 mg lysozyme powder was added for every ml of cell suspension. It was mixed well by vortexing and incubated at 0°C for 10 -15 minutes. Six millilitres of Triton buffer was added. It was mixed by rocking and rolling the centrifuge tube for 5 minutes. The cells lysed and became somewhat viscous and lighter in colour. In cases where the cells did not lyse properly, they were heated to 65°C for 5 min. The lysed cells were centrifuged for 45 minutes at 30,000 x g in Sorvall centrifuge. The supernatant was decanted into a small beaker and kept on ice. Appropriate amount of CsCl (1 g/ml of lysate solution) was dissolved in the lysate solution. When the CsCl was completely dissolved, 0.2 ml of ethidium bromide solution (20 mg/ml) was mixed. The lysate was transferred to ultracentrifuge tubes (Beckman quickseal, 5/8 x 3 in), the tubes filled with paraffin oil and heat sealed. The tubes were centrifuged in Beckman ultracentrifuge (18-80) in a 80Ti rotor at 60 K for 20 h at 15°C. The tubes were examined through vertically projected U.V. light. Plasmid appeared at the bottom of the two bands. The top band, if any, was the contaminating nuclear DNA. The plug was removed from the cap before puncturing the tube below the bottom of the two bands with a

hypodermic needle (21 G 1½). The DNA band was withdrawn by hypodermic and put in a small wide mouth test tube. One millilitre of CsCl-saturated isopropanol was added and the test tube was shaken gently for about 1 min. The phases were allowed to separate and the top phase removed and discarded. The procedure was repeated until the colour was gone from the plasmid aqueous phase. The DNA solution was transferred to a double knotted dialysis bag and dialysed against Tris-EDTA (TE) buffer (500 ml) overnight. The buffer was changed the next day and dialysis was continued for 2 - 3 h. The plasmid solution was transferred in siliconized Eppendorf tubes and stored at -20°C.

Comment

The widespread use of bacterial plasmids in recombinant DNA research has led to the development of many plasmid DNA extraction and purification methods (Maniatis et al., 1982; Birnboim et al., 1979; Holmes and Quigley, 1981; Zasloff et al., 1978).

In order to offset the cost of CsCl, the rapid alkaline extraction method for the isolation of plasmid DNA (Birnboim, 1983) was tried but it was found that there was genomic DNA contamination. Since fairly pure plasmid DNA was required, this method was abandoned in favour of the CsCl centrifugation method. The CsCl method gave us 250 - 450 µg plasmid DNA per liter of cell culture. The

DNA was sufficiently pure to be used for nick translation and isolation of myc specific fragment.

VI Isolation and Purification of Oncogene Insert from Plasmid

Many methods to recover DNA from agarose gels have been developed (Southern, 1975; reviewed by Wu et al., 1976; Smith, 1970) but none of them is completely satisfactory. The following method has worked well in our laboratory.

(a) Method

The plasmid was digested with the appropriate restriction enzyme such that the insert was cut out of the plasmid. This sample was then run on an agarose gel (1%) overnight at 30V. In order to stain the DNA, 0.5 µg/ml ethidium bromide was added to the 0.5X TBE (Tris-Borate EDTA) running buffer. The insert was localized using a U.V. lamp and excised with a sharp knife. About 0.5 cm more was removed from the sides in the gel where the band once was in order to make a trough larger than the band width. Enough buffer was removed from the gel apparatus such that it was just below the gel. The buffer inside the trough was also removed. A sheet of dialysis tubing was placed on the trough such that it covered at least 1 cm over all edges of the trough. Gel strips were placed around the trough to form a "box". The gel strip containing the insert was placed in the dialysis tubing covered trough and 300 - 500 µl of running buffer (without EtBr) was added to it. The gel was run at 150V for 1.5 - 2 hours until DNA (EtBr stained) could no longer be visualized by the U.V. lamp in the gel band. The

insert was electroeluted into the dialysis bag in the trough. The buffer containing the insert was collected in 1.5 ml siliconized Ependorf tubes. The trough was rinsed with 100 - 300 µl more buffer. The polarity was then reversed and gel was run at 150V for 30 - 60 secs. The buffer was removed and collected in the Eppendorf tube. Three times the volume of isoamyl alcohol (water saturated) was added to the Eppendorf tube and vortexed. The tubes were spun in the microfuge for a minute. When the two phases separated, the top isoamyl alcohol layer was discarded. The procedure was repeated 5 - 6 times until all of the ethidium bromide was removed. An equal volume of 1:1 phenol-choloroform mixture was added to the aqueous phase. Phenol was saturated with Tris-EDTA (TE) buffer, pH 8.0 while chloroform was a 24:1 mixture of chloroform and isoamyl alcohol respectively. It was vortexed well and spun in a Beckman micro-centrifuge for 5 min at 4°C. The top aqueous layer was carefully removed, ignoring the interface material which had agarose particles, and re-extracted with 1:1 phenol:chloroform. Sodium acetate, pH 5.2 was added to the aqueous phase for a final concentration of 0.3 M. Two times the volume of absolute ethanol was added and DNA was precipitated at -20°C overnight. The DNA was pelleted in Beckman micro-centrifuge for 30 minutes in the cold room. The supernatant was discarded and the pellet was dried in a freeze-dryer. An appropriate amount of sterile water was added and the DNA pellet was dissolved by placing the Eppendorf tube in 37°C water bath for an hour.

Comment

There have been several methods developed to recover DNA from agarose gels (Wu et al., 1976). There are two main difficulties involved in the recovery of DNA from agarose gels. First, most grades of agarose contain sulfated polysaccharides which inhibit many enzymes such as restriction endonuclease and polymerases. Second, the amount of DNA recovered depends on its molecular weight (Maniatis et al., 1982). The smaller fragments such as 1 Kb are recovered well whereas fragments greater than 20 Kb in size are rarely recovered.

Several methods were tried unsuccessfully to recover DNA from agarose gels. The method of electroelution of DNA into the dialysis bag and the recovery of DNA from low melting temperature agarose (Maniatis et al., 1982) gave poor yield. However, one cannot state that these methods do not work since they have worked well in other laboratories (Maniatis et al., 1982). By using the method of electroelution of DNA into a trough containing dialysis bag, a DNA yield of 20 - 80% was obtained.

VII Rapid Estimation of DNA Concentration Using Ethidium Bromide (EtBr) Agarose Plate Method

Plastic petri plates containing 15 ml of 1% agarose and 0.5 µg/ml of EtBr were prepared. Five microliter of DNA solution whose concentration was to be determined was spotted on the agarose plate. Equal volume of DNA of known concentration was spotted in an ordered array from about 25 µg/ml to 250 µg/ml. The spots were allowed to soak

into the agarose overnight. The intensities were compared by photography with short wavelength U.V. light. The DNA concentration was determined by comparing the intensity of DNA solution whose concentration was to be determined with intensity of DNA of known concentration.

VIII Restriction Endonuclease Digestion of DNA

(a) Restriction Endonucleases

The restriction enzymes used in this study were obtained from BRL (Bethesda Research Labs), Boeringer and P. L. Biochemicals.

(b) Restriction Enzyme Buffers

The buffers specific for each enzyme were prepared as outlined by the companies from which the enzymes were obtained. High, medium and low salt buffers were also used. The components of these buffers were as follows:

Low Salt Buffer: (10 mM Tris (pH 7.4), 10 mM $MgSO_4$, 1 mM DTT)

Medium Salt Buffer: (50 mM NaCl, 10 mM Tris (pH 7.4), 10 mM $MgSO_4$, 1 mM DTT)

High Salt Buffer: (100 mM NaCl, 50 mM Tris (pH 7.4), 10 mM $MgSO_4$)

(c) Method

A typical reaction mixture consisted of an appropriate amount of DNA, 1x restriction enzyme buffer, restriction enzyme, 0.1% BSA (bovine serum albumin) and water. In general, 1 unit of enzyme cleaves 1 µg of DNA in 15 minutes. Two to three units of enzyme was used per µg of genomic DNA. It was mixed well and incubated at 37°C for an appropriate time,

depending on whether partial or complete digestion was desired. After incubation, 20 µl of stop solution was added per 50 µl of the reaction mixture to inactivate restriction enzymes. The composition of the stop solution was 4 M urea, 50% sucrose, 50 mM EDTA, 0.1% bromophenol blue, pH 7.

(d) Agarose Gel Electrophoresis

The agarose gel electrophoresis was conducted in a horizontal configuration using a BRL (Bethesda Research Labs) apparatus. Electrophoresis grade agarose used was from BRL (No. 5510). Agarose (0.7% - 1.0%) was dissolved in electrophoresis buffer by bringing to a boil in a microwave oven. Tris-Borate EDTA electrophoresis (TBE) buffer (89 mM Tris-OH, 89 mM Boric acid, 2.5 mM Na_2 EDTA, pH 8.3) was used since it is a high capacity buffer electrolyte known to give sharp bands. It was made sure that the solution was homogeneous and there were no solid particles of agarose remaining. The solution was cooled to 60°C before pouring the gel. The sample wells were made with a lucite comb inserted into the molten gel. The gel was completely submerged underneath (about 1 mm) the electrophoresis buffer (0.5 x TBE) containing 0.5 µg/ml ethidium bromide. A voltage gradient of about 1V/cm was applied for 16 - 18 h.

(e) Photography

Photographs of the gels were made by illuminating the gel with incident U.V. light and recording on Polaroid Type 667 film.

IX Southern Blotting

In 1975, Southern developed a method for transfer of DNA from agarose gels to nitrocellulose paper by capillary action. During the course of our study, this method was employed on nitrocellulose paper, gene screen and gene screen plus membranes.

(a) Transfer of DNA from Agarose Gels to Nitrocellulose Paper

The method employed has been described by Maniatis et al. (1982) in Molecular Cloning, a laboratory manual.

(b) Transfer of DNA from Agarose Gels to Gene Screen

Gene screen is a hybridization transfer membrane (Catalog No. NEF-972) developed by New England Nuclear Company. The protocol followed was as described by the company in their instruction manual.

(c) Transfer of DNA from Agarose Gels to Gene Screen Plus

Gene screen plus is a hybridization transfer membrane (Catalog No. NEF-976) also developed by New England Nuclear Company. Southern blot procedure was followed as per the instruction manual of the company.

X Nick Translation

(a) Method

In a siliconized Eppendorf centrifuge tube, the following reactants were placed in the specified order:

(i) dCTP-alpha-^{32}P (obtained from NEN) . . . 7.0 µl (70 µ Ci)

(ii) dTTP-alpha-^{32}P (obtained from NEN) . . . 7.0 µl (70 µ Ci)

(iii) dCTP (Sigma) . . . . . 0.5 µl (0.1 mM)

(iv) dTTP (Sigma) . . . . . 0.5 µl (0.1 mM)

(v) dGTP (Sigma) . . . . . 4.0 µl (0.1 mM)
dATP

(vi) Plasmid DNA or insert (0.25 - 0.5 µg
DNA final concentration) . . . . . 2 - 10 µl

(vii) Soln II (polymerase I, DNase I;
obtained from Amersham Nick
Translation Kit N5000) . . . . . 3.0 µl

The total volume was about 30 µl.

In cases, where only one radioactive nucleotide was used, the reactants were placed in the following order:

(i) dCTP-alpha-^{32}P (obtained from NEN) . . . 7.0 µl (70 µ Ci)

(ii) dCTP (Sigma) . . . . . 0.5 µl (0.1 mM)

(iii) dGTP (obtained from Amersham Nick
dATP Translation Kit N5000) . . . . . 4.0 µl
DTTP

(iv) Plasmid DNA or insert (0.25 - 0.5 µg
DNA final concentration) . . . . . 1 - 5 µl

(v) Soln II (polymerase I, DNase I;
obtained from Amersham Nick
Translation Kit N5000) . . . . . 2.0 µl

(vi) Translation grade water . . . . . 1.5 - 5.5 µl

Total volume was 20 µl.

The reactants were mixed and then centrifuged for 30 seconds in a Beckman micro-centrifuge. The Eppendorf tube with the reactants was incubated at 16°C (water bath in cold room) for 90 minutes. The reaction was stopped by adding nick translation stop buffer (0.02 m Na_2 EDTA, 2 mg/ml sonicated salmon sperm DNA, 0.2% SDS) and 4 µg/µl tRNA. The Eppendorf tube was centrifuged for 30 seconds in a Beckman micro-centrifuge and incubated at 68°C for 15 minutes.

A 5 ml column of Sephadex G-50 was prepared in 5 ml

disposable plastic pipette plugged at the tip with siliconized glass wool. Plastic and siliconized glass wool are essential since small amounts of DNA may be lost by adherence to glass surfaces. The column was not allowed to run dry and was topped when necessary with dilute buffer (1 mM Tris.Cl, 0.1 mM EDTA, pH 7.5). The column was set up behind glass shield with all the necessary equipment and Geiger counter for monitoring radioactivity. Lead apron was worn during all manipulations with ^{32}P. The entire reaction mix was added to the top of the Sephadex column collecting the effluent into a suitable Eppendorf tube. The column was not allowed to run dry and was replenished with more buffer (1 mM Tris.Cl, 0.1 mM EDTA, pH 7.5). A Geiger counter was used to follow the position of the leading peak of radioactivity which was the labeled DNA. When this reached the bottom of the column, it was collected in a separate Eppendorf tube (volume was about 1 - 1.5 ml).

Determination of Radioactivity

The volume of the nick translated DNA solution was estimated and 5 µl of this was counted. Five microlitres of the nick translated DNA was placed in an Eppendorf tube and 5 µl bovine serum albumin (10 mg/ml in NaOH) and 100 µl trichloro acetic acid (20%) was added to precipitate the DNA. The Eppendorf tube was centrifuged for 30 sec in a Beckman micro-centrifuge. The precipitated solution was filtered through a millipore filter (0.45 µm) and washed with several volumes of 20% trichloroacetic acid. The filter was placed in

scintillation vial and 5 ml scintillant added. It was vortexed and the radioactivity was determined in a liquid scintillation counter (LS-230 Beckman).

Sample Calculation

Count = 155,000

Since used 5 µl of nick translated sample

Total volume of nick translated sample = 1500 µl

Concentration of plasmid used = 250 ng

Therefore, count per 250 ng = $\frac{155,000 \times 1500}{5} = 46 \times 10^6$ cpm

The count is 1.86×10^8 cpm per microgram of plasmid DNA.

XI Genomic Hybridization

(a) Solutions

17.5% sarkosyl
20 x SCP (2 M NaCl, 0.6 M Na_2HPO_4; 0.02 M Na_2 EDTA, pH 6.2)
4.4 SSC (0.66 M NaCl; 0.066 M Na acetate, pH 7.0)
5 mg/ml sonicated salmon sperm DNA
40x Denhardt's(8 g BSA type V, 8 g PVP 360, 8 g Ficoll 400; per liter)

Prehybridization

The nitrocellulose blot was first soaked in deionized water and then in 4.4 x SSC at room temperature for a minute or two. The nitrocellulose blot was placed in a suitable plastic bag with approximately 120 ml of prehybridization solution. All the bubbles were elminated and the bag was sealed. The prehybridization solution contained the following

50 ml (20 x SCP)
8.6 ml (17.5% sarkosyl)
3 ml (5 mg/ml sonicated salmon sperm DNA; boiled for 5 - 10 minutes prior to adding it)

37.5 ml (40 x Denhardt's)
50.9 ml distilled water

The prehybridization solution was filtered through Whatman No. 1 filter before adding sarkosyl and salmon sperm DNA. The solution was degassed for at least half an hour prior to use. The bag was then incubated in a 55°C shaking water bath for at least 3 h. In some instances, prehybridization was done overnight.

Hybridization

Two grams Dextran sulphate was dissolved in 10 - 20 ml of prehybridization solution. This solution was warmed to 55°C to help dissolve Dextran sulphate. This hybridization solution was degassed very thoroughly (0.5 - 1.0 hour). The bag with the prehybridizing nitrocellulose blot was removed from the 55°C shaking water bath and the prehybirdization solution was discarded. The 10 - 20 ml of the hybridization solution was poured in the bag and all air bubbles squeezed out. The probe was boiled for about 10 min. and added to the hybridizing solution in the bag. The bag was sealed and incubated in a 55°C shaking water bath. Hybridization was allowed to proceed for 24 - 42 h.

Washing

After hybridization, the blot was removed from the bag and washed twice for 15 min. in 200 ml of 6.6 SCP and 1% sarkosyl solution. All the washings took place at 55°C in the shaking water bath.

(b) Solutions

20 x SSC
40 x Denhardt's
10% SDS
5 mg/ml salmon sperm DNA

Prehybridization

The nitrocellulose was soaked in 6 x SSC at room temperature for a few minutes. The nitrocelllulose blot was placed in an appropriate plastic bag with approximately 30 ml of prehybridization solution. All the bubbles were eliminated and the bag was sealed. The prehybridization solution contained the following:

15 ml (20 x SSC)
5 ml (40 x Denhardt's)
0.5 ml (10% SDS)
10 ml (5 mg/ml salmon sperm DNA; boiled 5 - 10 minutes prior to adding it)
19.5 ml water

Total volume was 50 ml.

The prehybridization solution was filtered through Whatman No. 1 filter before adding SDS and salmon sperm DNA. The solution was degassed for about 30 minutes. The bag was incubated in 55°C shaking water bath for at least 3 h.

Hybridization

The bag with the nitrocellulose blot was removed from the 55°C shaking water bath and the prehybridization solution was decanted. Then 10 ml of the new prehybridization solution was added to the bag and all the air bubbles were eliminated. The boiled probe was added to the bag. The bag was sealed and placed in a 55°C shaking water bath for 24 - 42 hours for

hybridization to take place.

Washing

The blot was removed from the bag after hybridization and washed in the following order:

(a) 2 x SSC and 1% SDS at room temperature for 20 minutes

(b) 2 x SSC and 1% SDS at 55°C for 20 minutes

(c) 1 x SSC and 1% SDS at room temperature for 20 minutes

(d) 1 x SSC and 1% SDS at 55°C for 20 minutes

(e) 0.5 x SSC and 1% SDS at room temperature for 20 minutes

(f) 0.5 x SSC and 1% SDS at 55°C for 20 minutes

(c) Hybridization of DNA to gene screen plus membrane was accomplished by following the protocol contained in the instruction manual of the New England Nuclear Company (Catalog No. NEF-976).

XII Autoradiography

We used Kodak X-omat R film. The folders used were 8 x 10 inch AQ exposure holder. The intensifying screens used were Kodak x-omatic cassettes.

XIII. The c-myc and v-myc probes

The probes used included pLMC-41 plasmid containing human c-myc oncogene incorporated in pBR 322, c-myc fragment from pLMC-41 plasmid and v-myc fragment from plasmid myc 3 Pst. The probes were labeled by nick translation as described and used to hybridize to blots with DNA extracted from Achlya.

9) p Myc 3 Pst plasmid (Vennstrom et al., 1981)

Oncogene: v-myc

Isolated from: Myelocytomatosis virus 29 genome.

Name of host organism: E. coli HB101

The plasmic was constructed by inserting mcv genome cloned in lambda gt we's; clone cut with Pst I, 1.5 kb fragment (myc specific) subcloned into pBR 322.

Plasmid description

(i) Size

myc specific fragment = 1.5 kb

pBR 322 = 4.3 kb

Total size + 5.8 kb.

(ii) Genetic markers

Tet^r Amp^s

(iii) Restriction sites

Pst I cleaves myc specific fragment of 1.5 kb out of the pBR 322 plasmid.

b) pLMC-41 plasmid (Dalla-Favera et. al., 1982)

proto-oncogene: c-myc

Name of host organism: E. coli HB101

Plasmid description

(1) Size

myc specific fragment = 8.2 kb

pBR322 = 4.3 kb

Total size = 12.5 kb.

(ii) Genetic markers

Tet^s Amp^r

(iii) Restriction sites

EcoRl/Hind III digest cleaves 8.2 kb c-myc fragment out of the pBR322 plasmid.

RESULTS

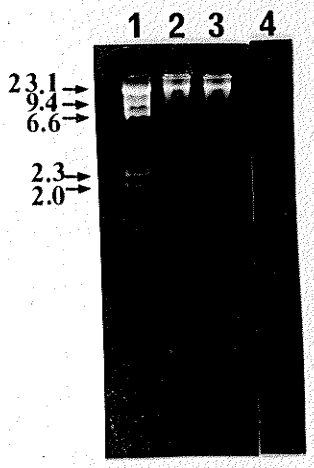

FIG.1

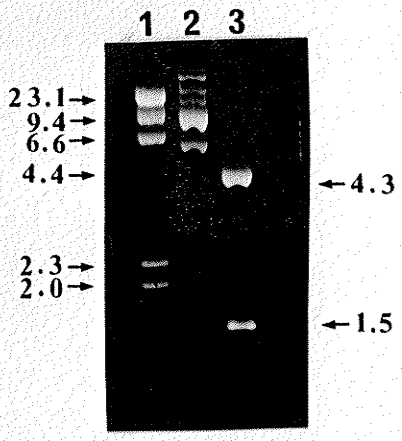

FIG.2

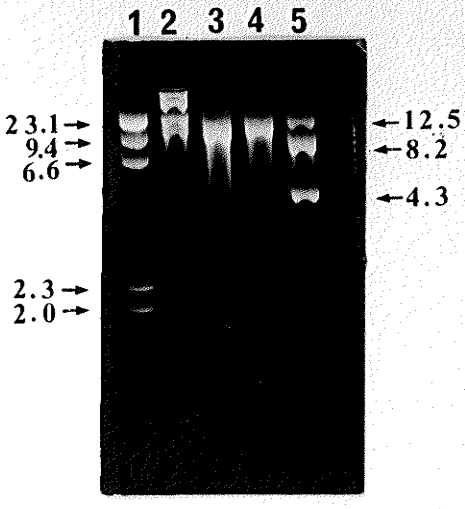

FIG.3

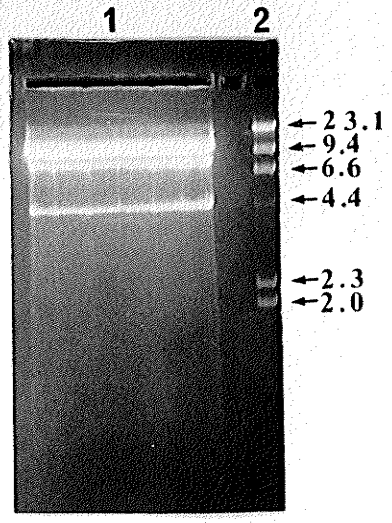

FIG.4

*Figure 1

Gel electrophoresis of the samples of the Achlya genomic DNA. Five microgram of genomic DNA digested with 10 mg of DNase-free RNase (Well 3) and 2.5 µg of DNase (Well 4) was electrophoresed in 1% agarose along with undigested DNA (Well 2) as a control. The samples were run at 36 volts for 16 hours. The λ Hind III cut fragments were run as markers in Well 1; with the arrows and numbers indicating the size of each band in kilobase pairs.

Figure 2

Characterization of Myc 3 Pst plasmid DNA by gel electrophoresis. Two micrograms of Myc 3 Pst plasmid was electrophoresed in 1% agarose after the following treatment: Well (2): Uncut plasmid, Well (3): plasmid cleaved with Pst 1, and Well (1): λ Hind III cut fragments were run as markers. The arrows and numbers indicate the size of each band in kilobase pairs. The samples were run at 36 volts for 16 hours. Untreated plasmid migrates as multiple bands since closed circular, nicked circular and linear DNA of same molecular weight migrate through agarose gel at different rates.

Figure 3

Characterization of pLMC-41 plasmid DNA by gel electrophoresis. Two micrograms of pLMC-41 plasmid was electrophoresed in 1% agarose after the following treatment: Well (2): Uncut plasmid, Well (3): plasmid cleaved with EcoRI, Well (4): plasmid cleaved with Hind III, Well (5): plasmid cleaved with EcoRI and Hind III, and Well (1): λ Hind III cut fragments were run as markers. The arrows and numbers indicate the size of each band in kilobase pairs. The samples were run at 36 volts for 16 hours.

Figure 4

Recovery of c-myc specific fragment from agarose gels. Forty-five micrograms of p41 plasmid containing c-myc oncogene was cleaved with EcoRI and Hind III and electrophoresed in 1% agarose gel at 30 volts for fourteen hours (Well 1). In Well 2, λ Hind III cut fragments were electrophoresed as markers; arrows and numbers indicate the size of each band in kilobase pairs.

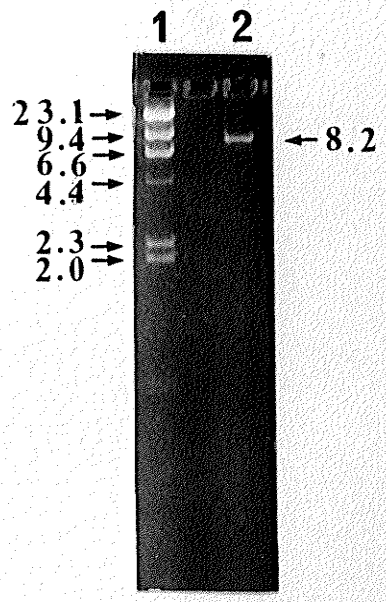

FIG.5

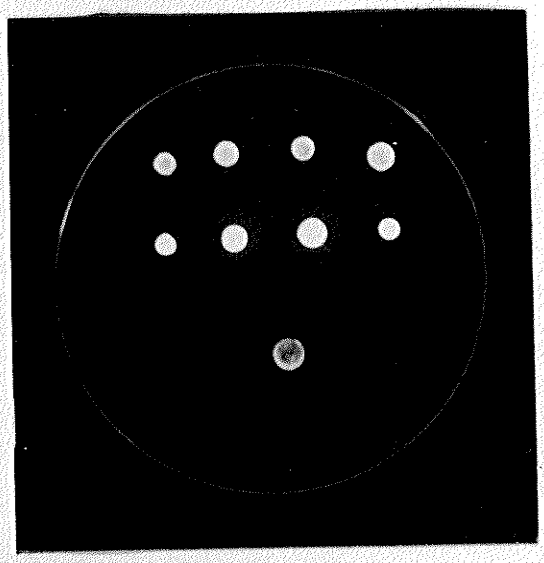

FIG.6

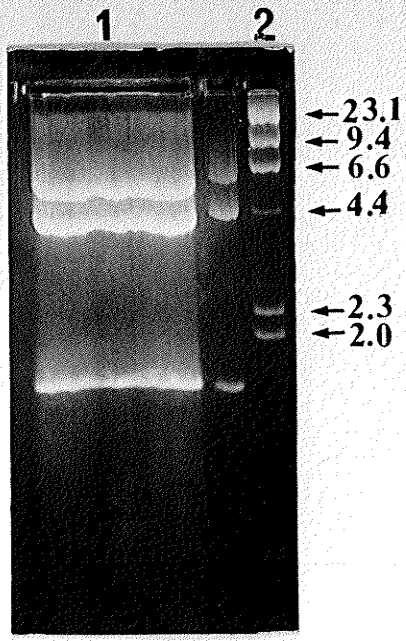

FIG.7

Figure 5

Gel electrophoresis of a sample of c-myc DNA insert recovered from agarose gel. Five microliters of the 8.2 kilobase DNA fragment recovered from plasmid pLMC-41 was electrophoresed in 1% agarose gel and run at 30 volts for 14 hours. Well (1): λ Hind III cut fragments as markers and Well (2): the recovered c-myc fragment of size 8.2 kilobase pairs. The arrows and numbers indicate the size of DNA bands in kilobase pairs.

Figure 6

Rapid estimation of DNA concentration using ethidium bromide (Agarose plate method). Five microliters of the c-myc DNA fragment,(pooled) whose concentration was to be determined, was applied on agarose plate containing 0.5 μg/ml ethidium bromide. Five microliters of λ DNA of known concentration was applied alongside. First row (left to right): 25 μg/ml λ DNA, 50 μg/ml λ DNA, 75 μg/ml λ DNA and 100 μg/ml λ DNA. Second row (left to right): 125 μg/ml λ DNA, 150 μg/ml λ DNA, 200 μg/ml λ DNA and 250 μg/ml λ DNA. Third row: c-myc fragment. The concentration of the c-myc fragment appeared to be less than 25 μg/ml.

Figure 7

Recovery of the v-myc specific fragment from agarose gel. One hundred micrograms of myc 3 Pst plasmid containing v-myc oncogene was cleaved with Pst I and electrophoresed in 1% agarose gel at 30 volts for 14 hours (Well 1). In Well 2, λ Hind III cut fragments were electrophoresed as markers. The arrows and numbers indicate the size of each band in kilobase pairs.

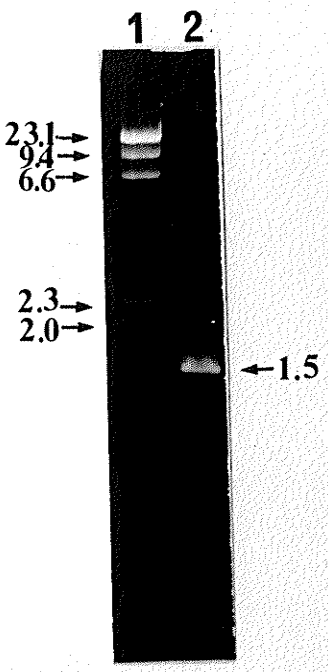

FIG. 8

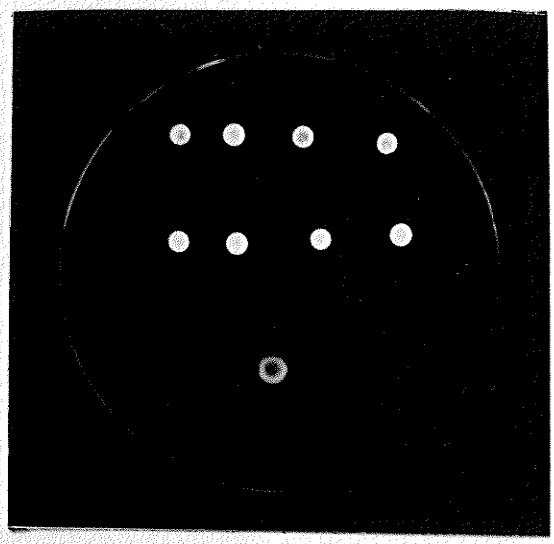

FIG. 9

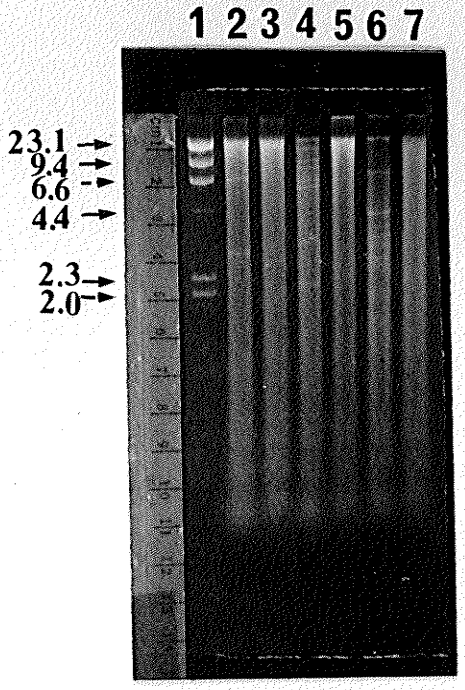

FIG. 10

Figure 8

Gel electrophoresis of a sample of v-myc DNA insert recovered from agarose gel. Five microliters of the 1.5 kilobase DNA fragment recovered from plasmid Myc 3 Pst was electrophoresed in 1% agarose gel and run at 30 volts for 14 hours. Well (1): λ Hind III cut fragments as markers and Well (2): the recovered v-myc fragment of size 1.5 kilobase pairs. The arrows and numbers indicate the size of DNA bands in kilobase pairs.

Figure 9

Rapid estimation of DNA concentration using ethidium bromide (Agarose plate method). Five microliters of the v-myc DNA fragment, whose concentration was to be determined, was applied on agarose plate containing 0.5 μg/ml ethidium bromide. Five microliters of λ DNA of known concentration was applied alongside. First row (left to right): 25 μg/ml λ DNA, 50 μg/ml λ DNA, 75 μg/ml λ DNA and 100 μg/ml λ DNA. Second row (left to right): 125 μg/ml λ DNA, 150 μg/ml λ DNA, 200 μg/ml λ DNA and 250 μg/ml λ DNA. Third row: v-myc fragment. The concentration of the v-myc fragment appeared to be less than 25 μg/ml.

Figure 10

Gel electrophoresis of samples of the Achlya genomic DNA digested with various restriction endonucleases. Five micrograms of Achlya DNA was digested with the indicated restriction endocucleases, electrophoresed in 1% agarose and transferred to nitrocellulose paper. Restriction endonuclease digestions were as follows: Lane (2), $K_{pn}I$, Lane (3) Sal I, Lane (4) EcoR1, Lane (5) Xho I, Lane (6) Hind III and Lane (7) Bam HI. The gel was run at 36 v for 16 hours. λ Hind III cut fragments were run as markers in lane (1). The arrows and numbers indicate the size of DNA bands in kilobase pairs. The experiment was conducted to get different restriction patterns with various restriction enzymes. This was done to get different hybridization patterns with different restriction enzymes in order to determine the probability of putative myc copy number in Achlya.

Figure 11

Blot hybridization analysis of the Achlya genome with myc probe. Achlya DNA was digested with the indicated restriction endonucleases, electrophoresed in 0.5% agarose gel, transferred to nitrocellulose and hybridized with pLMC-41 plasmid probe nick translated to 1.85 x 10^8 cpm per microgram. Seven and a half microgram of Achlya DNA was loaded in each channel: Lane (1) λ Hind III digest, Lane (2) KpnI digest and Lane (3) Sal I digest. The arrows and numbers indicate the size of DNA bands in kilobase pairs. The hybridization and washing were carried out at 55°C.

Figure 12

Blot hybridization analysis of the Achlya genome with myc probe. Achlya DNA was digested with the indicated restriction endonuclease, electrophoresed in 0.7% agarose gel, transferred to nitrocellulose and hybridized with pLMC-41 plasmid probe nick translated to 9.4 x 10^7 cpm per microgram. Five microgram of Achlya DNA was loaded in the channel and restriction digested with Bam HI. The arrow and number indicates the size of DNA band in kilobase pairs. The hybridization and washing were carried out at 55°C.

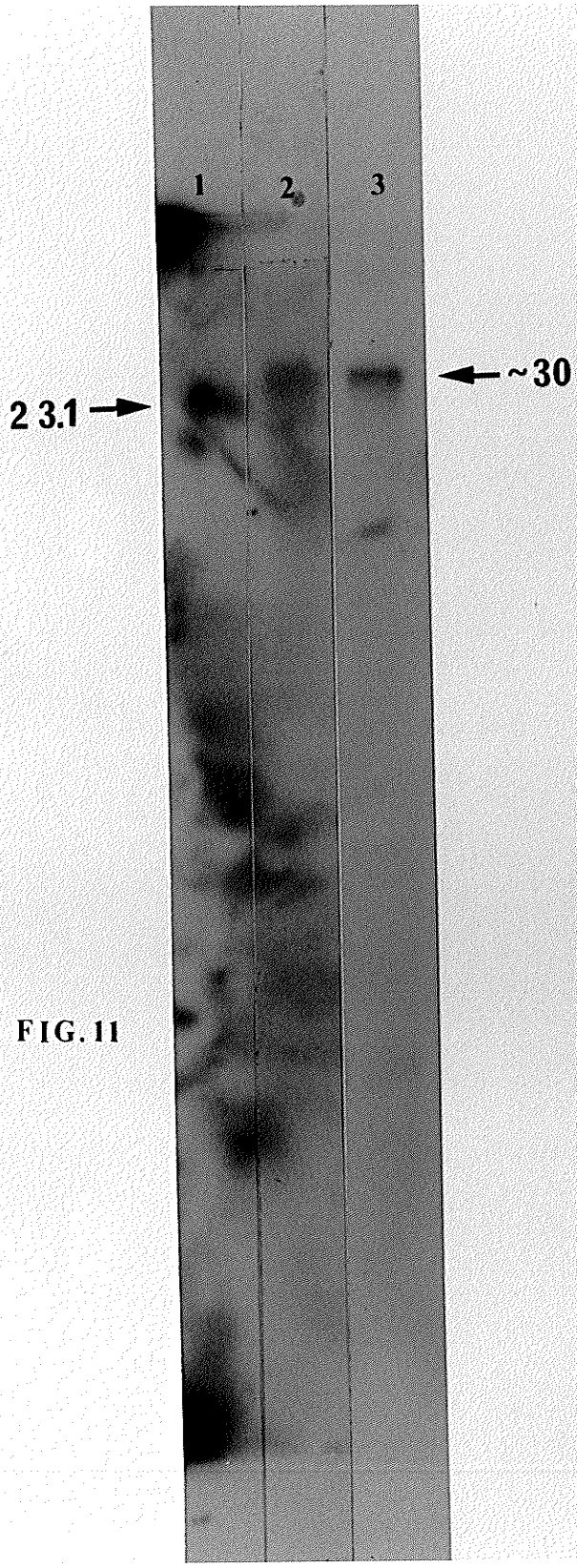

FIG. 11

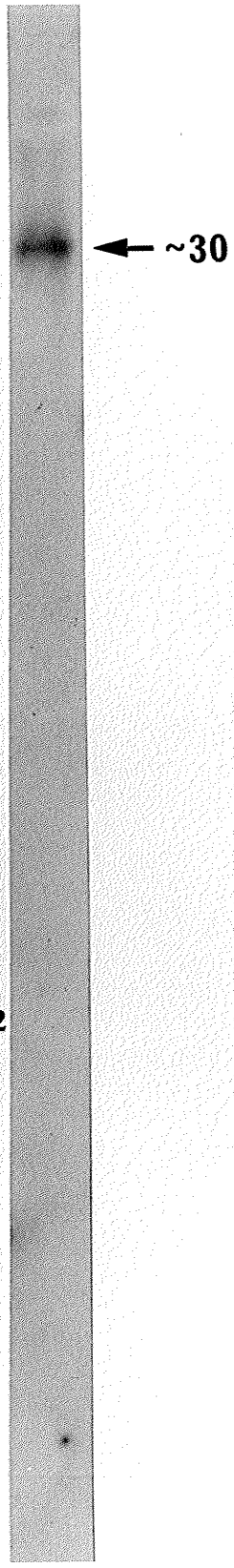

FIG. 12

Figure 13

Blot hybridization analysis of the Achlya genome with myc probe. Achlya DNA was digested with the indicated restriction endonucleases, electrophoresed in 0.5% agarose gel, transferred to nitrocelluose and hybridized with pLMC-41 plasmid probe nick translated to 2 x 10^8 cpm per microgram. Nine microgram of Achlya DNA was loaded in each channel: Lane (1) $K_{pn}I$ digest, Lane (2) PvuI digest, Lane (3) Bam HI digest and Lane (4) Sal I digest. The arrow and number indicates the size of DNA band in kilobase pairs. The hybridization and washing were carried out at 55°C.

Figure 14

Blot hybridization analysis of the Achlya genome with myc probe. Achlya DNA was digested with the indicated restrictions endonucleases, electrophored in 0.5% agarose gel, transferred to nitrocellulose and hybridized with pLMC-41 plasmid probe nick translated to 7.2 x 10^7 cpm per microgram. Six microgram of Achlya DNA was loaded in each channel: Lane (1), Kpn I digest and Lane (2), Sal I digest. The arrow and number indicates the size of DNA band in kilobase pairs. The hybridization and washing were carried out at 55°C.

Figure 15

Blot hybridization analysis of the Achlya genome with pBR322 probe. Achlya DNA was digested with the indicated restriction endonucleases, electrophoresed in 0.5% agarose gel, transferred to nitrocellulose and hybridized with pBR322 probe nick translated to 2.8 x 10^8 cpm per microgram. Seven-and-a-half microgram of Achlya DNA was loaded in each channel: Lane (1) λ Hind III digest, Lane (2) KpnI digest, Lane (3), KpnI digest, Lane (4) KpnI digest, Lane (5) Sal I digest, Lane (6) Sal I digest and Lane (7) Sal I digest. The arrows and numbers indicate the size of DNA bands in kilobase pairs. The hybridization and washing were carried out 55°C.

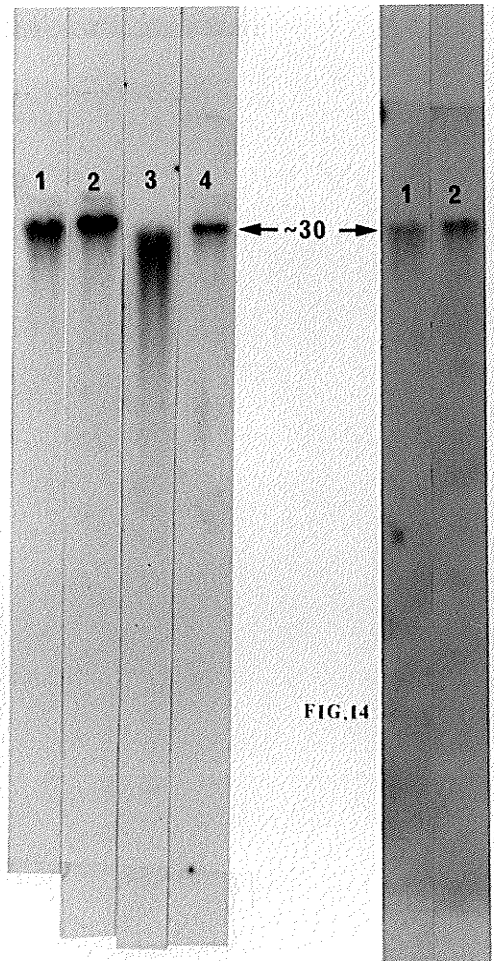

FIG. 13

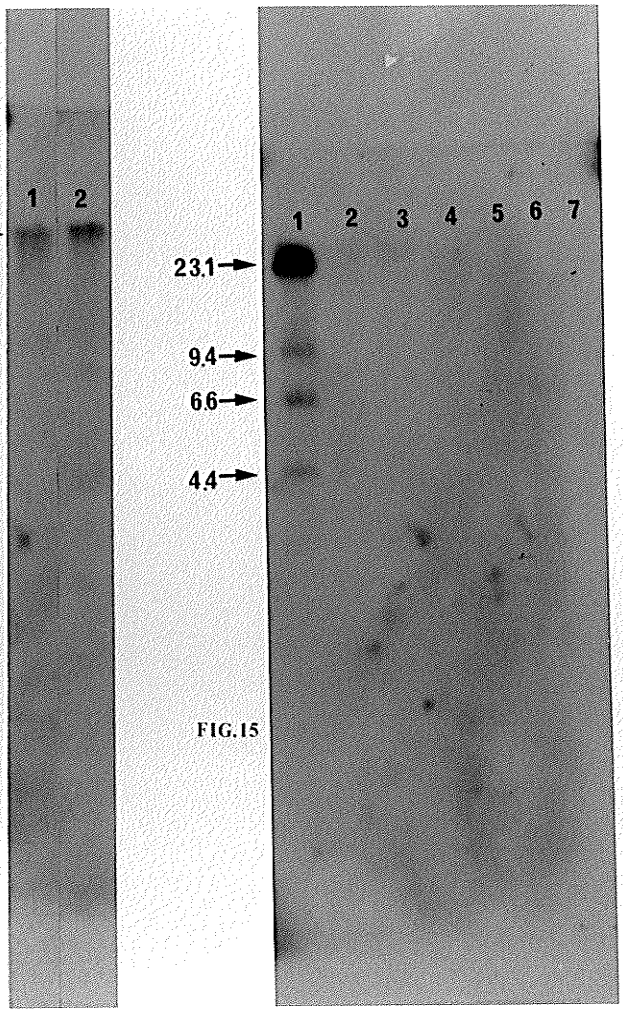

FIG. 14

FIG. 15

Figure 16

Blot hybridization analysis of the Achlya genome with myc probe. Achlya DNA was digested with the indicated restriction endonucleases, electrophoresed in 0.5% agarose gel, transferred to gene screen plus and hybridized with c-myc fragment nick translated to 3.5×10^7 cpm per microgram. Six microgram of Achlya DNA was loaded in each channel: Lane (1) KpnI digest, Lane (2) KpnI digest, Lane (3) Sal I, Lane (4), Sal I and Lane (5) Sal I. The arrow and number indicates the size of DNA band on kilobase pair. The hybridization and washing were carried out at 55°C.

Figure 19

Blot hybridization analysis of rat genome with myc probe. Rat DNA was digested with the indicated restriction endonucleases, electrophoresed in 0.7% agarose gel and transferred to gene screen plus and hybridized with v-myc fragment nick translated to 2.4×10^8 cpm per microgram. Five microgram of rat DNA was loaded in each channel: Lane (1) KpnI digest, Lane (2) Hind III digest and Lane (3) EcoRI digest. The hybridization and washing were carried out at 55°C.

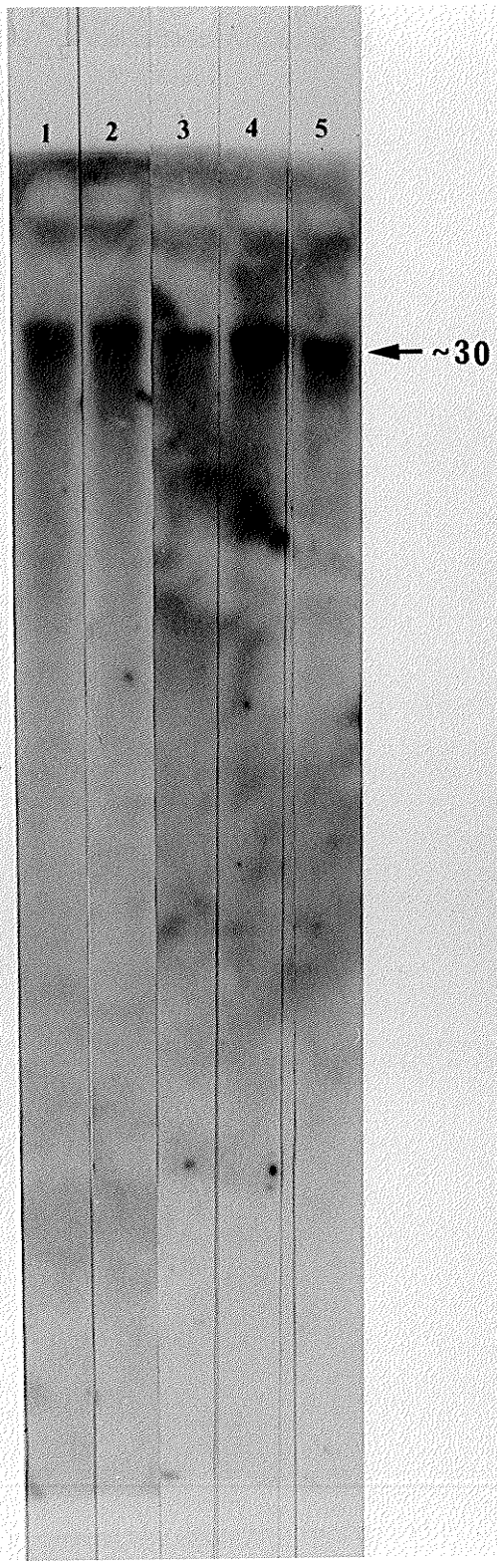

FIG. 16

FIG. 19

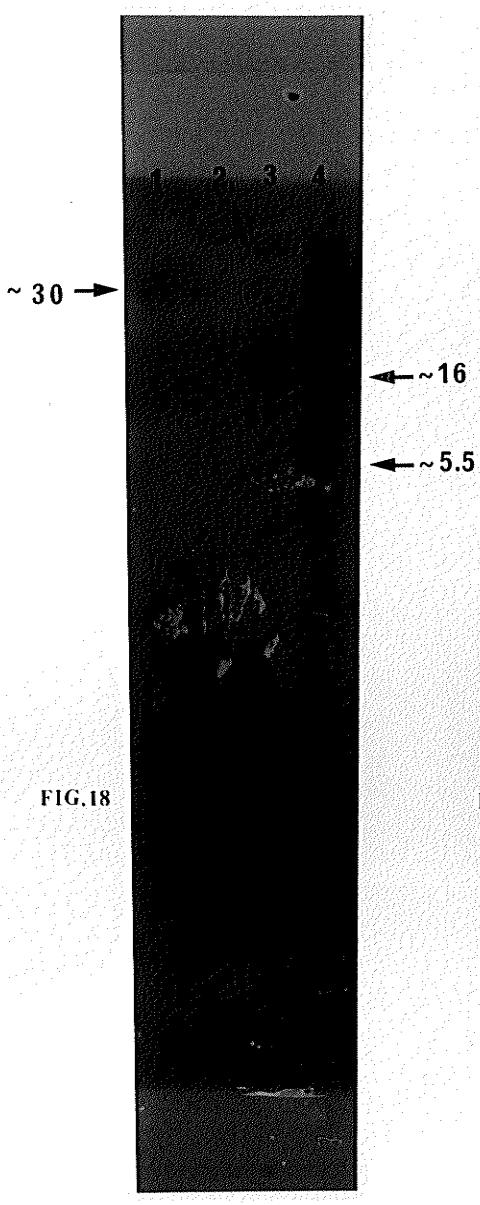

FIG.18

FIG.19

FIG.20

Figure 18

Blot hybridization analysis of the Achlya genome with myc probe. Achlya DNA was digested with the indicated restriction endonucleases, electrophoresed in 0.7% agarose gel, transferred to nitrocellulose and hybridized with pLMC-41 plasmid probe nick translated to 1.9×10^8 cpm per microgram. Seven-and-a-half microgram of Achlya DNA was loaded in each channel: Lane (1) to Lane (4) KpnI digest. The arrows and numbers indicate the size of DNA band in kilobase pairs. They hybridization and washing were carried out at 55°C.

Figure 17

Blot hybridization analysis of the Achlya genome with myc probe. Achlya DNA was digested with the indicated restriction endonucleases, electrophoresed in 0.7% agarose gel, transferred to gene screen plus and hybridized with v-myc fragment nick translated to 2.4×10^8 cpm per microgram. Twenty microgram of Achlya DNA was loaded in each channel: Lane (1) KpnI digest, Lane (2) Hind III and Lane (3) EcoRI digest. The arrows and numbers indicate the size of DNA band in kilobase pairs. The hybridization and washing were carried out at 55°C.

Figure 20

Blot hybridization analysis of rat genome with myc probe. Rat DNA was digested with the indicated restriction endonuclease, electrophoresed in 0.7% agarose gel, transferred to nitrocellulose and hybridized with v-myc fragment probe nick translated to 6.6×10^8 cpm per microgram. Five microgram of rat DNA was loaded in the channel and restriction digested with Bam HI. The arrow and number indicates the size of DNA band in kilobase pairs. The hybridization and washing were carried out at 55°C.

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I. Isolation and purification of Achlya genomic DNA

Achlya genomic DNA was isolated and purified by procedure described in materials and methods. Several batches of the Achlya genomic DNA, varying in concentration from 80 to 300 µg/ml, were isolated and purified to obtain sufficient quantity for the subsequent experiments. A low concentration DNA batch was concentrated by ethanol precipitation and dissolved in small aliquots of water.

Achlya genomic DNA was tested for purity by subjecting small samples of DNA (5 µg) to digestion with DNase (2.5 µg) and RNase (10 mg) and nothing (control) which were then run on agarose gel. The Hind III cut fragments were run on the gel as markers. Figure 1 shows that when the sample was treated with RNase (well 3) or nothing (well 2), the DNA bands disappeared. The intensity of the DNA bands in wells 2 and 3 was comparable and hence essentially free of RNA contamination. The approximate size of the DNA isolated appeared to be larger than 23.0 kb when compared to the Hind III cut fragments (well 1).

II. Isolation and purification of plasmid DNAs

A CsCl-gradient ultracentrifugation method was employed to isolate plasmid DNA. The CsCl method yielded 250-450 µg of plasmid DNA per titer of cell culture.

The Myc 3 Pst plasmid DNA was characterized for purity by gel electrophoresis (figure 2). When the untreated (control) Myc 3 Pst plasmid DNA was run on the agarose gel, several bands appeared (well 2). This was observed because untreated plasmid migrates

as multiple bands since closed circular, nicked circular and linear DNA of the same molecular weight migrate through agarose gel at different rates. The digestion of the Myc 3 Pst plasmid DNA with Pst 1 enzyme (well 3) produced two DNA bands representing the 4.3 kb pBR 322 fragment and the 1.5 kb v-myc fragment. The Pst 1 restriction enzyme cleaves the myc specific fragment of 1.5 kb out of the pBR322 plasmid. The appearance of a faint 5.8 kb band (well 3) represents the linear form of Myc 3 Pst plasmid. The other faint bands (well 3) represent closed circular and nicked circular DNA of the Myc 3 Pst plasmid.

The pLMC-41 plasmid DNA was also characterized by gel electrophoresis (figure 3). When untreated (control) pLMC-41 plasmid DNA was run on the agarose gel, several bands appeared (well 2). These bands represented untreated plasmid which migrates as multiple bands since closed circular, nicked circular and linear DNA of the same molecules weight migrates through agarose gel at different rates. The digestion of the pLMC-41 plasmid with EcoRI (well 3) and Hind III (well 4) gave a band of 12.5 kb size. This band represented the linear form of the pLMC-41 plasmid since both EcoRI and Hind III restriction enzymes cut only once into the plasmid. When the pLMC-41 plasmid was digested with EcoRI/Hind III enzymes were observed. The 8.2 kb band represents the c-myc fragment while the 4.3 kb band was the pBR322 fragment. The EcoRI/Hind III enzyme digest cleaves the 9.2 kb c-myc fragment out of the pBR322 plasmid. The 12.5 kb band represents the linear form of the pLMC-41 plasmid indicating that the digestion was incomplete.

III. Recovery and purification of c-myc DNA insert

The recovery and purification of DNA fragments from DNA bands separated on agarose gels was done as described in the materials

and methods. The yield of the DNA insert varied from 2% to 10%. Figure 4 shows the gel electrophoresis of a sample of c-myc DNA insert recovered from the agarose gel. The 8.2 kb fragment represents the c-myc oncogene which was recovered. The 4.3 kb fragment was the pBR 322 plasmid. The 12.5 kb band represented the linear form of the pLMC-41 plasmid indicating that the digestion was incomplete.

The sample of c-myc DNA insert recovered from agarose gel was tested for presence and purity by gel electrophoresis. Figure 5 shows the gel electrophoresis of a sample of c-myc DNA insert recovered from agarose gel. The 8.2 kb c-myc oncogene migrated to its appropriate position. The absence of any other band indicated its purity.

IV. Rapid estimation of the concentration of c-myc DNA fragment

The estimation of the concentration of the c-myc fragment recovered from the agarose gel was using ethidium bromide as described in materials and methods. Figure 6 shows that the concentration of the c-myc DNA fragment was estimated by comparing its fluorescent intensity to the intensities of a series of DNA of known concentration. Figure 6 shows that the concentration of the c-myc fragment appeared to be less that 25 µg/ml.

V. Recovery and purification of v-myc insert

The recovery and purification of the v-myc DNA insert was done in a similar way as described previously with the c-myc fragment. Figure 7 shows the gel electrophoresis of a sample of v-myc DNA insert recovered from the agarose gel. The 1.5 kb fragment represents the v-myc oncogene which was recovered. The 4.3 kb fragment was the pBR 322 plasmid. The 5.8 kb band represented the linear from the Myc 3

Pst plasmid indicating that the digestion with restriction enzyme Pst 1 was incomplete.

The sample of the v-myc DNA insert recovered from the agarose gel was tested for presence and purity by gel electrophoresis. Figure 8 shows the gel electrophoresis of a sample of v-myc DNA insert recovered from agarose gel. The 1.5 kb v-myc oncogene migrated to its appropriate position. The absence of any other band also indicated its purity.

VI. Rapid estimation of the concentration of v-myc DNA fragment

The concentration of the recovered v-myc DNA fragment was estimated using ethidium bromide agarose plate method described in materials and methods. Figure 9 shows that the concentration of the v-myc DNA fragment was estimated by comparing its fluorescent intensity to the intensities of a series of DNA of known concentrations. Figure 9 shows that the concentration of the v-myc fragment appeared to be less than 25 µg/ml.

VII. Gel electrophoresis

Achlya genomic DNA was cleared with various restriction enzymes prior to electrophoresis and Southern transfer. The enzymes used included: Kpn I, Bam Hl, Pst 1, Xhol, Sal I, Cla I, Hpa I, Pvu I, Hind III, Xba I and EcoRI. Figure 10 shows electrophoresis of the Achlya genomic DNA after being digested with restriction enzymes (Kpn I, Sal I, EcoRI, XhoI, Hind III and Bam HI). The experiment was conducted to get different restriction patterns with various restriction enzymes. This was done to get different hybridization patterns with different restriction enzymes in order to determine the probability of putative myc copy number in Achlya. The restriction enzymes used were six base

cuters. The reasons for using these restriction enzymes are as follows:

(i) The six base cutting restriction enzymes would cleave the Achlya DNA into large fragments. The myc oncogene sequences have been found to be 8 kilobases in human DNA (Dalla-Favera et al., 1982) and 21 kilobases in the Drosophila DNA (Shilo and Weinberg, 1981). Therefore, it was expected that the putative myc oncogene would be of similar size in the Achlya genome.

(ii) The restriction sites within the human c-myc and the v-myc oncogene probes have been discovered. Based on this information, the restriction enzymes were selected such that some would cleave within the putative gene and some in the flanking regions. This would ensure that some DNA digests would have intact gene for hybridization purposes.

VIII. Hybridization of c-myc containing pBR322 plasmid (pLMC-41)

Southern blot analysis of Achlya DNA digested with Bam HI, Sal I, Pvu I and Kpn I, and probed with pLMC-41 plasmid are shown in figures 11, 12, 13 and 14. The probe containing c-myc fragment incorporated in pBR322 detected DNA fragments of about 30 kilobases in the Achlya genome. The 30 kilobase fragments were detected with blots of Achlya DNA cleaved with KpnI and Sal I (Figure 11); Bam Hi (Fig. 12); Kpn I, pvu I, Bam HI and Sal I (Fig 13); Kpn I and Sal I (Fig. 14). The hybridization could be accounted in several ways.

First, 0.5 - 0.7% agarose was used and large fragments cannot be resolved by 0.5 - 0.7% agarose. The hybridization pattern observed with various enzymes may represent large fragments of different sizes that cannot be resolved by 0.5 - 0.7% agarose.

Second, the Achlya DNA was not completely digested by the various

restriction enzymes. As a result, there were large fragments (30 kilobases) of undigested DNA. The observed hybridization could be explained by the phenomenon of non-specific adsorption where the probe got trapped by large amount of undigested DNA (Dr. LeJohn, personal communication). Third, it could be that the fragment homologous to c-myc was still in the undigested DNA. Therefore, the observed hybridization was due to the probe hybridizing the DNA fragment homologous to it.

If the first explanation held true, one could say that there are no Bam HI, Sal I, Pvu I or Kpn I restriction sites within the putative myc gene and instead the restriction sites are in the flanking regions. This is consistent with the fact that there are no Bam HI, Sal I, Pvu I or Kpn I restriction sites within the c-myc gene of the pLMC-41 plasmid probe. However, if there are restriction site(s) for these enzymes within the putative gene, they would be at extreme ends. The explanation that the fragment homolgous to c-myc was still in the undigested DNA seemed most plausible.

IX. Hybridization of pBR322 to Achlya genomic DNA

The results obtained by the hybridization of c-myc containing pBR322 plasmid to Achlya genomic DNA could not be explained by the reactivity of pBR322 plasmid DNA sequences that were present in the probe. Various results point to this conclusion.

First, an independent investigator working in the same laboratory at the same time hybridized pT 24-C3 plasmid to Achlya genomic DNA (Tjong, 1985). The pT-24-C3 is a human bladder ras oncogene incorporated into Bam HI site of pBR322 (Santos et. al., 1982). The resulting

hybridization pattern was totally different to the result obtained with the pLMC-41 plasmid. Second, analysis of Achlya DNA cleaved with Kpn I and Sal I restriction enzymes yielded no hybridization with pBR322 as a probe (Fig. 15). However, pBR322 did hybridize to Hind III cut fragments of size 23.2 kb, 9.4 kb, 6.6 kb and 4.4 kb showing there is some homology between pBR322 and Hind III fragments. This hybridization is due to binding of the "ori" region of the pBR322 plasmid and a portion of DNA that is homologous to it. Third, using c-myc fragment as a probe against Achlya DNA blot, we observed hybridization pattern that was similar to the one observed using the whole pLMC-4I plasmid (Fig. 16). Analysis of Kpn I and Sal I cleaved Achlya DNA yielded hybridization of a fragment greater than 23 kilobases. The explanation for hybridization of such large fragments is as stated previously.

X. Hybridization of v-myc fragment to Achlya genomic DNA

The probes used to detect distantly related sequences are successful only if a limited degree of sequence divergence has occurred during the evolution of these genes. Excessive sequence divergence results in less stable hybrids and consequently result in reduction of hybridization signal. The c-myc gene contains three coding regions and two intervening sequences (Saito et al., 1983). The presence of intervening sequences in the c-myc probe may have been hindering hybridization; resulting in less stable hybrids and thus the faint signal observed. Many previous studies have used retrovirus derived onc probes to search for homologous sequences in the DNA of variety

of species (Shilo and Weinberg, 1981). Based on these considerations, it was decided that v-myc probe be used to search for homologous sequences in the Achlya genomic DNA. A factor that would compensate for a weak hybridization signal observed previously with c-myc probe was taken into consideration. The lower sequence complexity of Achlya genome made it possible to apply a greater number of genome equivalents of DNA to each gel channel used in the Southern analysis. Instead of using 5 - 10 µg of DNA, approximately 10 - 20 µg of DNA was used per well (Fig. 17). This was done to proportionally enhance any signal detected by hybridization. The analysis of Kpn I, Hind III and EcoRI cleaved Achlya DNA using v-myc fragment as a probe detected DNA fragments of about 30 kilobase in the Achlya genome cleaved with Kpn I and Hind III (Fig. 17). The explanation for this phenomenon is as stated previously. However, analysis of an EcoRl cleaved Achlya DNA yielded two fragments that hybridized to the v-myc fragment probe. One fragment was greater than 23 kilobases while the other fragment was 14 kilobases in size. The hybridization signals were of weak intensity.

XI. Enzyme concentration effect on digestion of Achlya DNA

In view of overcoming the problem of hybridization to the large clump of DNA migrating slowly at the top of the gel, enzyme-concentration effect on digestion of Achlya DNA was looked at (Fig. 18). The concentration of DNA was kept constant while increasing the concentration of restriction enzyme Kpn I. (Lane 1: 5 units Kpn I/µg of DNA, Lane 2: 10 units Kpn I/µg of DNA, Lane 3: 15 units KpnI/µg of DNA, Lane 4: 20 units Kpn I/µg of DNA. As the concentration of restriction enzyme Kpn I increased, the DNA became completely digested and there was

disappearance of hybridization of 30 kilobase fragment. Instead, a faint hybridization of approximately 16 kilobase fragment was detected with the pLMC-41 probe. As the concentration of restriction enzyme Kpn I was increased further, a faint hybridization of 5.5 kilobase fragment was detected. This observation can be explained by the Kpn I restriction sites closer to the gene being cleaved as the concentration of Kpn I increased.

This explains why the 30 kilobase fragment was cleaved to 16 kilobases; which in turn was further cleaved to 5.5 kilobases.

Hybridization of v-myc fragment to rat genomic DNA

The hybridization of v-myc fragment to rat genomic DNA served a two-fold purpose. First, it was intended as a control. Second, a comparison could be made of homology between v-myc probe and the putative myc in rat to the homology between v-myc probe and the putative myc gene in Achlya. During the first attempt, analysis of Kpn 1, Hind III and EcoR1 cleaved rat DNA using v-myc fragment as a probe was conducted on the same blot (Fig. 19). The probing of Kpn I, Hind III and EcoRI cleaved rat DNA with v-myc fragment resulted in a hybridization smear. The most likely explanation of this observation is that the hybridization and washing conditions (55°C) were not stringent enough. In the second attempt, analysis of Bam HI and Hind III cleaved rat DNA using v-myc fragment as a probe detected DNA fragment of about 15 kilobases in the rat genome cleaved with Bam Hl (Fig. 20). The hybridization signal was of weak intensity and was comparble to the intensity observed with that of the putative myc oncogene band

observed in the Achlya genome cleaved with EcoRl (Figure 17). However, the blot with the Achlya genome cleaved with EcoRl (Figure 17) was exposed for 15 hours in intensifying screen compared to the blot of rat genome cleaved with Bam Hl, which was exposed for 6 hours in intensifying screen. Although in the case of Achlya DNA, 20 µg was applied to each channel, only 5 µg of rat genome was applied to each channel in this case. Therefore, it seems the homology between v-myc probe and the putative myc gene in rat is greater than the homology between v-myc probe and the putative myc gene in Achlya.

DISCUSSION

I wanted to obtain a broader view of the evolution of proto-oncogenes by determining whether these genes could be detected in organism as primitive as Achlya. The presence of these genes in non-vertebrate organism like Achlya would suggest a role of these genes in cellular metabolism. Eventually, one could then take advantage of the well-known life cycle of less complex organisms like Achlya to gain insights into the function and expression of proto-oncogenes at various stages.

The presence of putative myc gene in the Achlya genome DNA

The results obtained seem to indicate that sequence homologous to v-myc and c-myc may be present in the Achlya genome (Figs. 11, 12, 13, 14, 16, 18, 19). However, the hybridization signals were very weak and therefore, this indication can only be taken as preliminary evidence. In order to know whether Achlya genome contains myc gene, one will have to clone the hybridizing fragment, sequence it and finally look at its product. The contamination of the DNA sample with RNA did not pose any problem since the aim was to detect a putative myc oncogene in the Achlya genome. However, if one is to clone the hybridizing fragment in the future, one will have to be concerned about the problem of RNA contamination.

Also, recording to the evolutionary theory, the weak homology between Achlya putative myc and mammalian myc indicates that the putative myc of Achlya is excessively diverged from the mammalian ones. The exact degree of homology, however, cannot be decided based on the data gathered so far. Only a comparison of the nucleotide sequence will determine the accurate degree of homology. The experiments also indicated that the homology between v-myc probe

and the putative myc gene in rat is greater than the homology between v-myc probe and the putative myc gene in Achlya. Thus, further indicating that putative myc gene in Achlya may have diverged from the mammalian myc gene.

The probability of putative myc oncogene copy number

The hybridization analysis of Achlya genomic DNA with c-myc probe, where enzyme-concentration effect on digestion of Achlya DNA was looked at (see Fig. 18 and the results section) indicated one or more copies of putative myc gene may be present. The following conclusions can be derived from this experiment.

(a) Since 5.5 kilobases is the smallest single hybridization band detected, it implies that there may be one copy of the putative myc oncogene whose size must be smaller or equal to 5.5 kilobases.

(b) The possibility of having two or more copies of the putative myc oncogene cannot be overruled. It could be possible that the gene copies are arranged in tandem array fashion with similar flanking region such that when cleaved with Kpn I, the gene copies migrate to the same position (i.e. 5.5 kb).

(c) The myc oncogene in humans is known to code for approximately 55,000 dalton protein and the 5.5 kilobase fragment is sufficient to code for such a protein.

(d) The possibility of having two copies of the gene contained in the 5.5 kilobase fragment is unlikely because if it were so, then each gene would have a size equal or smaller than 2.75 kilobases. This size of gene is unlikely to code for 55,000 dalton protein.

(e) The 5.5 kilobase fragment should represent the intact gene or there may be Kpn I restriction site(s) at extreme end(s) of the gene.

The hybridization analysis of the Achlya genomic DNA using v-myc fragment as a probe (see Fig. 19 and the results section), gave the following probability of the putative myc oncogene copy number.

(a) There is a possibility that there is an EcoRl site within the putative myc oncogene such that there is only one copy of the gene. However, in comparison, there is no EcoRl site within the v-myc fragment used as a probe (Alitalo et. al., 1983; Vennstrom et. al., 1981).

(b) The possibility of having two copies of the putative myc oncogene cannot be discarded. If this is true, then the two observed hybridization bands should be of similar intensity. In fact, the two hybridizing bands appear to be of similar intensity.

(c) It may also be possible that there were other bands that could not be seen due to the high radioactive background. The high radioactive background could not be resolved. There may have been non-specific adsorption of probe to salmon sperm DNA (Dr. LeJohn, personal communication) or the hybridization conditions used were not stringent enough. The use of more stringent conditions provided no hybridizing bands.

CONCLUSION

There seems to be preliminary indication of sequences homologous to myc oncogene in the Achlya genome. In future, it may be possible to study the myc oncogene homolog by isolating this in the form of molecular clones from the Achlya genomic library. Such isolation would allow one to make a detailed study of the sequences. Recently, nucleotide sequence analysis in Drosophila have revealed sufficient homology to account for the observed hybridization between v-myc and the Drosophila clones but have failed to detect significant amino acid sequence homology (Madhavan et al., 1985). Therefore, it seems unlikely that the genomic sequences Madhavan et al. (1985) have isolated by hybridization with v-myc represent homologs of the vertebrate myc gene.

In future, one could isolate the messages and the life cycle of Achlya could be exploited to look at differential expression of the gene at various stages. A parallel work has been done in Drosophila where mRNA complementary to the v-myc and to the Drosophila genomic segments have been detected in embryos, pupae, adults and kc cells (Madhavan et al., 1985). Their results indicate early embryonic transcripts are of maternal origin and may play a crucial role in early development.

The study of the myc oncogene in a less complex organism like Achlya would significantly broaden the spectrum of organisms in which oncogene structure and function could be studied.

REFERENCES

REFERENCES

Abrams, H. D., Rohrscheider, L. R., and Eisenman, R. N. (1982). Nuclear location of the putative transforming protein of Avian Myelocytomatosis virus. Cell 29:427-439.

Adams, J., Gerondakis, S., Webb, E., Corcoran, L. M., and Cory, S. (1983). Cellular myc oncogene is altered by chromosome translocation to the immunoglobulin locus in murine plasmacytomas and is rearranged similarly in human Burkitt lymphomas. Proc. Natl. Acad. Sci. USA 80:1982-1986.

Alexopoulos, C. J. (1962). Introductory Mycology. 2nd Edn., John Wiley and Son Inc., New York, pp. 138-158.

Alitalo, K., Bishop, J. M., Smith, D. H., Chen, E. Y., Golby, W. W. and Levinson, A. D. (1983). Nucleotide sequence of the v-myc oncogene of avian retrovirus MC29. Proc. Natl. Acad. Sci. USA 80:100-104.

Alitalo, K., Schwab, M., Lui, C. C., Varmus, H. E., and Bishop, J. M. (1983). Homogeneously staining chromosomal regions contain amplified copies of an abundantly expressed cellular oncogene (c-myc) in malignant neuroendocrine cells from a human colon carcinoma. Proc. Natl. Acad. Sci. USA 80:1707-1711.

Banerji, J., Olson, L., and Schaffner, W. (1983). A lymphocyte-specific cellular enhancer is located downstream of the joining region of immunoglobulin heavy chain genes. Cell 33:729-740.

Barksdale, A. W. (1963). The uptake of exogenous Hormone A by certain strains of Achlya. Mycologia 55:164-171.

Baserga, R. (1983). Growth in size and cell DNA replication. Mol. Cell. Biol. 3(2):214-219.

Battey, J., Moulding, C., Taub, R., Murphy, W., Stewart, T., Potter, H., Lenoir, G., and Leder, P. (1983). The human c-myc oncogene: structural consequences of translocation into the IgH in Burkitt lymphoma. Cell 34:779-787.

Beard, J. W. (1980). Viral Oncology. Ed. Klein, G. (Raven)., New York, pp. 57-87.

Bernard, O., Cory, S., Gerondakis, S., Webb, E., and Adams, J. M. (1983). Sequence of the murine and human cellular myc oncogenes and two modes of myc transcription resulting from chromosome translocation in B lymphoid tumors. EMBO J. 2:2375-2383.

Beug, H., Van Kuchbach, A., Doderlein, G., Conscience, J. F., and Graf, T. (1973). Chicken hematopoietic cells transformed by seven

strains of defective avian leukemia viruses display three distinct phenotypes of differentiation. Cell 18:375-390.

Birnboim, H. C., and Doly, J. (1979). Rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acid Res. 7(6):1513-1523.

Birnboim, H. C. (1983). A rapid alkaline extraction method for the isolation of plasmid DNA. Methods in Enzymology 100:243-255.

Bishop, J. M. (1981). Enemies within: The genesis of retrovirus oncogenes. Cell 23:5-6.

Bishop, J. M. (1983). Cellular Oncogenes and Retroviruses. Ann. Rev. Biochem. 52:301-354.

Bister, K., Hayman, M. J., and Vogt, P. K. (1977). Defectiveness of avian myclocytomatosis virus. MC29: Isolation of long-term non-producer cultures and analysis of virus-specific polypeptide synthesis. Virology 82:441-448.

Bister, K., and Duesberg, P. H. (1982). Advances in viral oncology. Vol. 1. Ed. Klein, G. (Raven)., New York, pp. 3-42.

Blair, D. G., Oskarrsson, M., Wood, T. G., McClements, W. L., Fischinger, P. J., and Vande Woude, G. G. (1981). Activation of the transforming potential of a normal cell sequence: A molecular model for oncogenesis. Science 212:941-943.

Cameron, L. E., and LeJohn, H. B. (1972). On the involvement of calcium in amino acid transport and growth of the fungus Achlya. J. Biol. Chem. 247:4729-4739.

Campisi, J., Gray, H. E., Pardee, A. B., Dean, M., and Sonenshein, G. E. (1984). Cell-cycle control of c-myc but not c-ras expression is lost following chemical transformation. Cell 36:241-247.

Capon, D. J., Chen, E. Y., Levinson, A. D., Seeburg, P. H., and Groeddel, D. V. (1983). Complete nucleotide sequences of the T24 human bladder carcinoma oncogene and its normal homologue. Nature 302:33-37.

Collett, M. S., Erikson, E., Purehio, A. F., Brugge, J. S., and Erikson, R. L. (1979). A normal cell protein similar in structure and function to the avian sarcoma virus transforming gene product. Proc. Natl. Acad. Sci. USA 76:3159-3163.

Collett, M. S., and Erikson, R. L. (1979a). Protein kinase activity associated with the avian sarcoma virus src gene product. Proc. Natl. Acad. Sci. USA 75:2021-2024.

Collett, M. S., Brugge, J. S., and Erikson, R. L. (1979b). Characterization of a normal avian cell protein related to the avian sarcoma virus transforming gene product. Cell 15:1363-1370.

Collett, M. S., Purchio, A. F., and Erikson, R. L. (1980). Avian sarcoma virus-transforming protein pp 60^{src} shows protein kinase activity specific for tyrosine. Nature 285:167-169.

Collins, S. J., and Groudine, M. (1982). Amplification of endogenous myc-related DNA sequences in a human myeloid leukemia cell line. Nature 298:679-681.

Collins, S. J., and Groudine, M. (1983). Rearrangement and amplification of c-Abl sequences in the human chronic myelogenous leukemia cell line K-562. Proc. Natl. Acad. Sci. USA 80:4813-4817.

Cooper, J. A., and Hunter, T. (1982). Discrete primary locations of a tyrosine protein kinase and of 3 proteins that contain phosphotyrosine in virally transformed chick fibroblasts. J. Cell. Biol. 94(2):287-290.

Cooper, J. A., Bowen-Pope, D. F., Raines, E., Ross, R., and Hunter, T. (1983). Similar effects of platelet-derived growth factor on the phosphorylation of tyrosine in cellular proteins. Cell 31:263-273.

Corcoran, L. M., Adams, J. M., Dunn, A. R., and Cory, S. (1984). Murine T lymphomas in which the cellular myc oncogene has been activated by retroviral insertion. Cell 37:113-122.

Cory, S., Gerondakis, S., and Adams, J. M. (1983). Interchromosomal recombination of the cellular oncogene c-myc with the immunoglobulin heavy chain locus in murine plasmacytomas is a reciprocal exchange. Embo. J. 2:697-703.

Dalla-Favera, R., Gelmann, E. P., Martinotti, S., Franchini, G., Papas, T. S., Gallo, R. C., and Wong-Staal, F. (1982). Cloning and characterization of different human sequences related to the onc gene (v-myc) of avian myelocytomatosis virus (MC29). Proc. Natl. Acad. Sci. USA 79:6497-6501.

Dalla-Favera, R., Wong-Staal, F., and Grallo, R. C. (1982a). One gene amplification in promyelocytic leukemia cell line HL-60 and primary leukemic cells of the same patient. Nature 299:61-63.

DeFeo, D., Gonda, M. A., Young, H. A., Chang, E. H., Lawry, D. R., Scolnick, E. M., and Ellis, R. W. (1981). Analysis of two divergent rat genomic clones homologous to the transforming gene of Harvey murine sarcoma virus. Proc. Natl. Acad. Sci. USA 78:3328-3332.

DeFeo-Jones, D., Scolnick, E. M., Koiler, R., and Dhar, R. (1983). Ras-related gene sequences identified and isolated from Saccharomyces cerevisiae. Nature 306:707-709.

DeFranco, A. L., Kung, J. T., and Paul, W. E. (1982). Regulation of growth and proliferation in B cell populations. Immunol. Rev. 64:161-182.

Dick, M. W. (1973). Saprolegniales. The Fungi, an advanced treatise (Ainswork, G. C., Sparrow, F. K., and Sussmann, S. S., eds.), Academic Press, New York, Vol. IVB:113-144.

Donner, P., Greiser-Wilke, I., and Moelling, K. (1982). Nuclear localization and DNA binding of the transforming gene product of avian myelocytomatosis virus. Nature 296:262-265.

Doolittle, R. F., Hunkapiller, M. W., Hood, L. D., Devare, S. G., Robbins, K. C., Aaronson, S. A., and Antoniades, H. N. (1983). Simian sarcoma virus oncogene v-sis, is derived from the gene (or genes) encoding a platelet derived growth factor. Science 221:275-277.

Duesberg, P. H., Bister, K., and Vogt, P. K. (1977). The RNA of avian acute leukemia virus MC29. Proc. Natl. Acad. Sci. USA 74:4320-4324.

Duesberg, P. H. (1980). Transforming genes of retroviruses. Cold Springs Harbor Symposium. Quant. Biol. 44(PI):13-29.

Ek, B., Westermark, B., Wasteson, A., and Heldin, C. H. (1982). Stimulation of tyrosine-specific phosphorylation by platelet derived growth factor. Nature 295:419-420.

Erikson, E., and Erikson, R. L. (1980). Identification of a cellular protein substrate phosphorylated avian sarcoma virus-transforming gene product. Cell 21:829-836.

Erikson, J., ar-Rushdi, H., Drwinga, H. L., Nowell, P. C., and Croce, C. M. (1983). Transcriptional activation of the translocated c-myc oncogene in Burkitt lymphoma. Proc. Natl. Acad. Sci. USA 80:820-824.

Franchini, G., Even, J., Sherr, C. J., and Wong-Staal, F. (1981). Onc sequences (v-fes) of Snyder-theilen feline sarcoma virus are derived from non-contiguous regions of a cat cellular gene (c-fes). Nature 290:154-157.

Frankel, A., and Fischinger, P. T. (1976). Nucleotide sequences in mouse DNA and RNA specific for Moloney sarcoma virus. Proc. Natl. Acad. Sci. USA 73:3705-3709.

Fung, Y. K., Lewis, W. G., Crittenden, L. B., and Kung, H-J. (1983). Activation of the cellular oncogene c-erb B by LTR insertion: Molecular basis for induction of erythroblastosis by avian leukosis virus. Cell 33:357-368.

Furth, M. E., Davis, L. J., Fleurdelys, B., and Scolnick, E. M. (1982). Monoclonal antibodies to the p21 products of the transforming gene of Harvey sarcoma virus and of the cellular ras family. J. Virol. 43:294-304.

Gallwitz, D., Donath, C., and Sander, C. (1983). A yeast gene encoding a protein homologous to the human c-has/bas proto-oncogene product. Nature 306:704-706.

Gillies, S. D., Morrison, S. L., Oi, V. T., and Tonegawa, S. (1983). A tissue-specific transcription enhancer element is located in the major intron of a rearranged immunoglobulin heavy chain gene. Cell 33:717-728.

Goff, S. P., Gilboa, E., Witte, O. N., and Baltimore, D. (1980). Structure of the Abelson murine leukemia virus genome and the homologous cellular gene: Studies with cloned viral DNA. Cell 22: 777-785.

Graf, T. (1973). Two types of target cells for transformation with Avian myelocytomatosis virus. Virology 54:398-413.

Graf, T., and Beug, H. (1978). Avian leukemia viruses. Interaction with their target cells in vivo and in vitro. Biochim. Biophy. Acta. 516:269-299.

Gruss, P., Dhar, R., and Khoury, G. (1981). Simian virus-40 tandem repeated sequences as an element of the early promoter. Proc. Natl. Acad. Sci. USA 78:943-947.

Hamlyn, P. H., and Rabbitts, T. H. (1983). Translocation joins c-myc and immunoglobulin gamma-1 genes in a Burkitt lymphoma revealing a third exon in the c-myc oncogene. Nature 304:135-139.

Hayday, A., Gillies, S., Saito, H., Wood, C., Wiman, K., Hayward, W., and Tonegawa, S. (1984). Activation of a translocated human c-myc gene by an enhancer in the immunoglobulin in heavy chain locus. Nature 307:334-340.

Hayward, W. S., Neel, B. G., and Astrin, S. M. (1981). Activation of a cellular onc gene by promoter insertion in ALV-induced lymphoid leukosis. Nature 296:475-480.

Hollis, G. F., Mitchell, K. F., Battey, J., Potter, H., Taub, R. A., Lenoir, G. M., and Leder, P. (1984). A variant translocation places the lambda immunoglobulin genes 3' to the c-myc oncogene in Burkitt lymphoma. Nature 307:752-755.

Holmes, D. S., and Quigley, M. (1981). A rapid boiling method for the preparation of bacteria plasmids. Anal. Biochem. 114(1):193-197.

Hu, S. F., Lai, M. M. C., and Vogt, P. K. (1979). The genome of avian myelocytomatosis virus MC29 analyzed by heteroduplex mapping. Proc. Natl. Acad. Sci. USA 76:1265-1268.

Huebner, R. J., and Todaro, G. J. (1969). Oncogene of RNA tumor viruses as determinants of cancer. Proc. Natl. Acad. Sci. USA 64:1087-1094.

Hughes, S. H., Payvar, F., Spector, D., Schinke, R. T., Robinson, H. L., Payne, G. S., Bishop, J. M., and Varmus, H. E. (1979). Heterogeneity of genetic loci in chickens: Analysis of endogenous viral and non viral genes by cleavage of DNA with restriction endonucleases. Cell 18:347-359.

Hughes, S. H., Vogt, P. K., Stubblefield, E., Robinson, H., Bishop, J. M., and Varmus, H. F. (1980). Organization of endogenous and exogenous viral and linked non-viral sequences. Cold Spring Harbor. Symp. Quant. Biol. 44:1079-1089.

Hunter, T., and Sefton, B. M. (1980). Transforming gene product of Rous sarcoma virus phosphorylates tyrosine. Proc. Natl. Acad. Sci. USA 77:1311-15.

Hunter, T., Sefton, R. M., and Cooper, J. A. (1981). In Protein Phosphorylation, Cold Spring Harbor Conferences on cell proliferation. Eds. Rosen, O. M., and Krebs, E. G. (Cold Spring Harbor Labs, Cold Spring Harbor. New York 8:1189-1202.

Hunter, T. (1983). Proteins of oncogenes. Sci. Am. 251(2):70-79.

Karess, R. E., and Hanafusa, H. (1981). Viral and cellular src genes contribute to the structure of recovered avian sarcoma virus transforming protein. Cell 24:155-164.

Kasuga, M., Zick, Y., Blithe, D. L.,, Cretlaz, M., and Kahn, C. R. (1982). Insulin stimulates tyrosine phosphorylation of the insulin receptor in a cell-free system. Nature 298:667-669.

Keath, E. J., Caimi, P. G., and Cote, M. D. (1984). Fibroblast lines expressing activated c-myc oncogenes are tumorigenic in nude mice and syngeneic animals. Cell 39:339-348.

Kelly, K., Cochran, B. H., Stiles, C. D., and Leder, P. (1983). Cell specific regulation of the c-myc gene by lymphocyte mitogens and platelet derived growth factor. Cell 35:603-610.

Kozak, M. (1978). How do eukaryotic ribosomes select initiation regions in messenger RNA. Cell 15:1109-1123.

Land, H., Parada, L. F., and Weinberg, R. A. (1983). Tumorigenic conversion of primary fibroblasts requires at least two co-operating oncogenes. Nature 304:596-602.

Langbeheim, H., Shih, T. Y., and Scolnick, E. M. (1980). Identification of a normal vertebrate cell protein related to the p21 src of Harvey murine sarcoma virus. Virology 106:292-300.

Leder, P. (1983). Translocations among antibody genes in human cancer. Science 222:765-771.

LeJohn, H. B., and Braithewaite, C. E. (1984). Heat and nutritional shock induced proteins of the fungus Achlya are different and under

independent transcriptional control. Can. J. Biochem. and Cell Biol. 62:837-846.

Levinson, A. D., Oppermann, H., Levintow, L., Varmus, H. E., and Bishop, J. M. (1978). Evidence that the transforming gene of avian sarcoma virus encodes a protein kinase associated with a phosphoprotein. Cell 15:561-572.

Levinson, A. D., Oppermann, H., Varmus, H. E., and Bishop, J. M. (1980). The purified product of the transforming gene of avian sarcoma virus phosphorylates tyrosine. J. Biol. Chem. 255:11973-11980.

Levinson, B., Khoury, G., Van de-Woude, G., and Giruss, P. (1982). Activation of SV40 genome by 72-base pair tandem repeats of Moloney sarcoma-virus. Nature 295:568-572.

Little, C. D., Lau, M. M., Carney, D. N., Gazdar, A. F., and Minna, J. D. (1983). Amplification and expression of the c-myc oncogene in human lung cancer cell lines. Nature 306:194-196.

Madhavan, K., Bilodeau-Wentworth, D., and Wadsworth, S. C. (1985). Family of developmentally regulated, maternally expressed Drosophila RNA species detected by a v-myc probe. Mol. Cell. Biol. 5(1):7-16.

Maguire, R. T., Robins, T. S., Thorgeirsson, S., and Heilman, C. A. (1983). Expression of cellular myc and mos genes in undifferentiated B cell lymphomas of Burkitt and non-Burkitt types. Proc. Natl. Acad. Sci. USA 80:1947-1950.

Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982). Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory. New York.

Marcu, K. B., Harris, L. J., Slaton, L. W., Erikson, J., Watt, R., and Croce, C. M. (1983). Transcriptionally active c-myc oncogene is contained within NIARD, a DNA sequence associated with chromosome translocations in B cell neoplasia. Proc. Natl. Acad. Sci. USA 80:519-524.

Mathey-Prevot, B., Hanafusa, H., and Kawai, S. (1982). A cellular protein is immunologically cross reactive with and functionally homologous to the Fujinami Sarcoma-virus transforming protein. Cell 28:897-906.

McCoy, M. S., Toole, J. J., Cunningham, J. M., Chang, E. H., Lowry, O. R., and Weinberg, R. A. (1983). Characterization of a human colon/lung carcinoma oncogene. Nature 302:79-81.

Mellon, P., Pawson, A., Bister, K., Martin, G. S., and Duesberg, P. H. (1978). Specific RNA sequences and gene products of MC29 avian acute leukemia virus. Proc. Natl. Acad. Sci. USA 75:5874-5878.

Milner, J., and Milner, S. (1981). SV40-53K antigen: A possible role of 53K in normal cells. Virology 112:785-788.

Moscovici, C., Alexander, R. W., Moscovici, M. G., and Vogt, P. K. (1978). in Avian RNA tumour viruses (ed. Barlati, S., and DeGuili-Morghen, C.) Piccin, Padua. pp. 45-56.

Muller, R., Bravo, R., Burckhardt, J., and Curran, T. (1984). Induction of c-fos gene and protein by growth factor precedes activation of c-myc. Nature 312:716-720.

Mushinski, J. F., Bauer, S. R., Potter, M., and Reddy, E. P. (1983). Increased expression of myc related oncogene mRNA characterizes most Balb/c plasmacytomas induced by pristane or Abelson murine leukemia virus. Proc. Natl. Acad. Sci. USA 80:1073-1077.

Mushinski, J. F., Potter, M., Bauer, S. R., and Reddy, E. P. (1983). DNA rearrangement and altered RNA expression of the c-myb oncogene in mouse plasmacytoid lymphosarcomas. Science 220:759-798.

Newbold, R. F., and Overell, R. W. (1983). Fibroblast immortality is a pre-requisite for transformation by EJ c-Ha-ras oncogene. Nature 304:648-651.

Nishikura, K., Abbas, R., Erikson, J., Watt, R., Rovena, G., and Croce, C. M. (1983). Differential expression of the normal and of the translocated human c-myc oncogenes in B cells. Proc. Natl. Acad. Sci. USA 80:4822-4826.

Nushimura, J., Huang, J. S., and Deuel, T. F. (1982). Platelet-derived growth factor stimulates tyrosine specific protein kinase activity in Swiss mouse 3T3 cell membranes. Proc. Natl. Acad. Sci. USA 79:4303-4307.

Oppermann, H., Levinson, A. D., Varmus, H. E., Levintow, L., and Bishop, J. M. (1979). Uninfected vertebrate cells contain a protein that is closely related to the product of the avian sarcoma virus transforming gene (src). Proc. Natl. Acad. Sci. USA 76:1804-1808.

Oskarrsson, M. K., McClements, W. L., Blair, D. S., Maizel, J. V., and Vande-Woude, G. F. (1980). Properties of a normal mouse DNA sequence (sarc) homologous to the src sequence of Moloney sarcoma virus. Science 207:1222-1224.

Parker, R. C., Varmus, H. E., and Bishop, J. M. (1981). The cellular homologue (c-src) of the transforming gene of Rous sarcoma virus: Isolating, mapping and transcriptional analysis of c-src and flanking regions. Proc. Natl. Acad. Sci. USA 78:5842-5846.

Pawson, T. (1983). Regulation of a ras-related protein development of Dictyostelium discodeum. Mol. Cell. Biol. 5(1):33-39.

Payne, G. S., Courtneidge, S. A., Crittenden, L. B., Fadly, A. M., Bishop, J. M., and Varmus, H. E. (1981). Analysis of avian

leukosis virus DNA and RNA in bursal tumors: viral gene expression is not required for maintenance of the tumor state. Cell 23:311-322.

Payne, G. S., Bishop, J. M., and Varmus, H. E. (1982). Multiple arrangements of viral DNA and an activated host oncogene in bursal lymphomas. Nature 295:209-215.

Pledger, W. J., Stiles, C. D., Antoniades, H. N., and Scher, C. D. (1978). An ordered sequence of events is required before BALB/c-3T3 cells become committed to DNA synthesis. Proc. Natl. Acad. Sci. USA 74:4481-4485.

Pledger, W. J., Hart, C. A., Locatell, K. L., and Scher, C. D. (1981). Platelet derived growth factor-modulated proteins: constitutive synthesis by a transformed cell line. Proc. Natl. Acad. Sci. USA 78:4358-4362.

Ponticelli, A. S., Whitlock, C. A., Rosenberg, N., and Witte, O. N. (1982). In vivo tyrosine phosphorylations of the Abelson virus transforming proteins are absent in its normal cellular homolog. Cell 29:953-960.

Purchio, A. F., Erikson, E., Collett, M. S., and Erikson, R. L. (1981). In Cold Spring Harbor conference on cell proliferation - protein phosphorylation. (ed. Krebs, E., and Rosen, O.). Cold Spring Harbor, New York, 8:1203-1215.

Rabbitts, T. H., Hamlyn, P. H., and Baer, R. (1983). Altered nucleotide sequence of a translocated c-myc gene in Burkitt lymphoma. Nature 306:760-765.

Radke, K., and Martin, G. S. (1979). Transformation by Rous sarcoma virus: Effects of src gene expression on the synthesis and phosphorylation of cellular polypeptides. Proc. Natl. Acad. Sci. USA 76:5212-5216.

Radke, K., Gilmore, T., and Martin, G. S. (1980). Transformation by Rous sarcoma virus: A cellular substrate for transformation - specific protein phosphorylation contains phosphotyrosine. Cell 21:821-828.

Ralston, R. R., and Bishop, J. M. (1984). The protein products of the myc and myb oncogenes and adenovirus E1A are structurally related. Nature 306:803-806.

Rettenmeier, C. W., Anderson, S. M., Riemen, M. W., and Hanafusa, H. (1979). Gag related polypeptides encoded by replication defective avian oncoviruses. J. Virol. 32:749-761.

Rigby, P. W. J., Dieckmann, M., Rhodes, C., and Berg, P. (1977). Labelling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA-polymerase I. J. Mol. Biol. 113(1):237-251.

Robins, T., Bister, K., Garon, C., Papas, T., and Duesberg, P. H. (1982). Structural relationship between a normal chicken DNA locus and the transforming gene of the avian acute-leukemia virus MC29. J. Virol. 41(12):635-642.

Rohrschneider, L. R., Eisenman, R. N., and Leitch, C. R. (1979). Identification of a Rous sarcoma virus transformation-related protein in normal and mammalian cells. Proc. Natl. Acad. Sci. USA 76:4479-4483.

Roussel, M. S., Saule, S., Lagron, C., Rammens, L., Beug, H., Graf, T., and Stekelin, D. (1979). Three types of viral oncogenes of cellular origin specific for haemopoietic cell transformation. Nature 281:452-455.

Ruley, H. E. (1983). Adenovirus early region 1A enables viral and cellular transforming genes to transform primary cells in culture. Nature 304:602-606.

Saito, H., Hayday, A. C., Wiman, K., Hayward, W. S., and Tonegawa, S. (1983). Activation of the c-myc gene by translocation: A model for translational control. Proc. Natl. Acad. Sci. USA 80:7476-7480.

Scolnick, E. M., Rands, E., Williams, D., and Parks, W. P. J. (1973). Studies on nucleic acid sequences of Kirsten sarcoma virus - model for formation of a mammalian RNA containing sarcoma-virus. J. Virol. 12:458-463.

Scolnick, E. M., and Parks, W. P. J. (1974). Harvey sarcoma virus: A second murine type C sarcoma virus with rat genetic information. J. Virol. 13:1211-1219.

Sefton, B. M., Hunter, T., and Beeman, K. (1980). Relationship of polypeptide products of the transforming gene of Rous sarcoma virus and the homologous gene of vertebrates. Proc. Natl. Acad. Sci. USA 77:2059-2063.

Shalloway, D., Zelenetz, A. D., and Cooper, G. M. (1981). Molecular cloning and characterization of the chicken gene homologous to the transforming gene of Rous sarcoma virus. Cell 24:531-541.

Sheiness, D., and Bishop, J. M. (1979). DNA and RNA from uninfected vertebrate cells contain nucleotide sequences related to the putative transforming gene of avian myelocytomatosis virus. J. Virol. 31:514-521.

Sheiness, D., Fanshier, L., and Bishop, J. M. (1980). Identification of nucleotide sequences which may encode the oncogenic capacity of avian retrovirus MC29. J. Virol 28:600-610.

Sheiness, D., Hughes, S., Varmus, H. E., Stubblefield, E., and Bishop, J. M. (1980). The vertebrate homolog of the putative transforming

gene of avian myelocytomatosis virus: Characteristics of the DNA locus and its RNA transcript. Virol. 105:415-424.

Shen-Ong, G. L. C., Keath, E. J., Piccoli, S. P., and Cole, M. D. (1982). Novel myc oncogene RNA from abortive immunoglobulin gene recombination in mouse plasmacytomas. Cell 31:443-452.

Shilo, B. -Z., and Weinberg, R. A. (1981). DNA sequences homologous to vertebrate oncogenes are conserved in Drosophila melanogaster. Proc. Natl. Acad. Sci. USA 78:6789-6792.

Smith, H. O. (1980). Recovery of DNA from gels. Methods in Enzymol. 65:371-380.

Southern, E. M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503-517.

Spector, D. H., Varmus, H. E., and Bishop, J. M. (1978). Nucleotide sequences related to the transforming gene of avian sarcoma virus are present in the DNA of uninfected vertebrates. Proc. Natl. Acad. Sci. USA 75:4102-4106.

Stanton, L. W., Watt, R., and Marcu, L. B. (1983). Translocation, breakage, and truncated transcripts of c-myc oncogene in murine plasmacytomas. Nature 303:401-406.

Steffen, D. (1984). Provirus are adjacent to c-myc in some murine leukemia virus-induced lymphomas. Proc. Natl. Acad. Sci. USA 81:2097-2101.

Stehelin, D., Varmus, H. E., Bishop, J. M., and Vogt, P. K. (1976). DNA related to the transforming gene(s) of avian sarcoma viruses is present in normal avian DNA. Nature 260:170-173.

Takeya, T., Hanafusa, H., Junghans, R., Ju, G., and Skalka, A. M. (1981). Comparison between the viral transforming gene (src) of recovered avian sarcoma virus and its cellular homologue. Mol. Cell. Biol. 1:1024-1037.

Taub, R., Kirsch, I., Morton, C., Lenoir, G., Swan, D., Tronick, S., Aaaronson, S., and Leder, P. (1982). Translocation of the c-myc gene into the immunoglobulin heavy chain locus in human Burkitt lymphoma and murine plasmacytoma cells. Proc. Natl. Acad. Sci. USA 79:7837-7841.

Taub, R., Moulding, C., Battey, J., Murphy, W., Vasicek, T., Lenoir, G. M., and Leder, P. (1984). Activation and somatic mutation of the translocated c-myc gene in Burkitt lymphoma cells. Cell 36:339-348.

Taylor, J. M. (1979). The isolation of eukaryotic messenger RNA. Ann. Rev. Biochem. 48:681-717.

Tjong, K. K. (1985). A preliminary evidence for the possible existence of ras oncogene-homologous sequence(s) in Achlya genomic DNA. Master of Science thesis, Department of Microbiology, University of Manitoba.

Tsuchida, N., Gilden, R. V., and Hatanka, M. (1974). Sarcoma-virus related RNA sequences in normal rat cells. Proc. Natl. Acad. Sci. USA 71:4503-4507.

Ushiro, H., and Cohen, S. (1980). Identification of phosphotyrosine as a product of epidermal growth factor activated protein-kinase in A-431 cell membranes. J. Cell. Biol. 255:8363-8365.

Vennstrom, B., Moscovici, C., Goodman, H. M., and Bishop, J. M. (1981). Molecular cloning of the avian myelocytomatosis virus genome and recovery of infectious virus by transfection of chicken cells. J. Virol. 39(2):625-631.

Vennstrom, B., Sheiness, D., Zabialski, J., and Bishop, J. M. (1982). Isolation and characterization of c-myc, a cellular homolog of the oncogene (v-myc) of avian Myelocytomatosis virus strain 29. J. Virol. 42:773-779.

Watson, D. K., Psallidopoulous, M. C., Samuel, K. P., Dalla-Favera, R., and Papas, T. S. (1983). Nucleotide sequence analysis of human c-myc locus, chicken homologue and myelocytomatosis virus MC29 transforming gene reveals a highly conserved gene product. Proc. Natl. Acad. Sci. USA 80:3642.

Watson, D. K., Reddy, E. P., Duesberg, P. H., and Papas, T. S. (1983b). Nucleotide sequence analysis of the chicken C-MYC gene reveals homologous and unique coding regions by comparison with the transforming gene of avian myelocytomatosis virus MC29, Delta-GAG-MYC. Proc. Natl. Acad. Sci. USA 80:2146-2150.

Watt, R. A., Shatzman, A. R., and Rosenberg, M. (1985). Expression and characterization of the human c-myc DNA binding protein. Mol. Cell. Biol. 5(3):448-456.

Webster, R. K., Hall, D. H., Heeres, J., Wick, C. M., and Brandon, D. M. (1970). Achlya klebsiana and Pythium species as primary causes of seed rot and seedling disease of rice in California. Phytopath. 60:964-968.

Weinberg, R. A. (1982a). Oncogenes of spontaneous and chemically-induced tumors. Adv. Cancer Res. 36:149-163.

Weinberg, R. A. (1982b). Fewer and fewer oncogenes. Cell 30:3-4.

Weiss, R. A., Teich, N. M., Varmus, H., and Coffin, J. M. The molecular biology of tumour viruses. Cold Spring Harbor Laboratory, New York.

Westin, E. H., Wong-Staal, F., Gelmann, E. P., Dalla-Favera, R., Papas, T., Lauteberger, J. A., Eva, A., Reddy, E. P., Tronick, S. R., Aaaronson, S. A., and Gallo, R. C. (1982). Expression of cellular homologs of retroviral onc genes in human hematopoietic cells. Proc. Natl. Acad. Sci. USA 79:2490-2494.

Zasloff, M., Ginder, G. D., and Felsenfeld, G. (1978). New method for purification and identification of covalently closed circular DNA-molecules. Nucleic Acids Res. 5(4):1139-1152.

Zbigniew, D., Evanson, D., Staiano-Coica, L., Sharpless, T., and Melamed, M. (1979). Correlation between cell cycle duration and RNA content. J. Cell. Physiol. 100:425-438.