THE DEVELOPMENTAL EXPRESSION OF THE DICTYOSTELIUM DISCOIDEUM RAS GENE, AND PRELIMINARY DETECTION OF A SECOND RAS-HOMOLOGOUS SEQUENCE IN ITS GENOME.

By

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ABSTRACT

The expression of a mammalian ras gene analog was previously found by Reymond et al. to be developmentally regulated in *Dictyostelium discoideum* using Northern analysis of strain AX-3 RNA (1984, Cell 39;141) and by Pawson et al. using specific immunoprecipitation of in vivo synthesized proteins from strain V12M2 (1985, Mol. Cell Biol. 5;33). Due to differences in the results of the two studies, it was decided to further examine ras expression by applying both protein and RNA techniques to a single strain of *Dictyostelium* V12M2. RNA samples from strain V12M2 cells at different stages of development were analyzed using Northern blotting. The same RNAs were translated in vitro, and the ras proteins synthesized were immunoprecipitated and analysed by polyacrylamide gel electrophoresis.

In agreement with the findings of Reymond et al. (1984, Cell 39;141), Northern analysis with the cDNA ras probe revealed that the highest levels of the 1.2 and 0.9 kb ras mRNAs were present in the total RNA of V12M2 cells at the pseudoplasmodial stage of development, and very little ras mRNA was present in early developing cells.

In contrast to the Northern analysis the greatest amount of ras protein was in vitro translated from the RNA of vegetative and 2 hour cells. Hence this work confirms in a single strain of *Dictyostelium* that the greatest amount of ras protein is synthesized at those developmental stages that contained the lowest levels of mRNA detectable by the cDNA probe. Possible reasons for this phenomena are discussed.
In vitro RNA translation was also used to study the relationship between the two ras proteins of 23 and 24 kd. The proteins did not appear to be derived from one another by degradation or by post-translational modification. This result suggested that the two ras proteins of strain V12M2 must be derived from two different mRNAs.

High stringency Southern blots of AX-3 DNA showed the expected restriction fragments detected by Reymond et al. (1984, Cell 39;141). Low stringency blots showed three faint additional restriction fragments in Eco RI digests of AX-3 DNA. No additional restriction fragments were generated by an Eco RI-Bgl II digest, but two of the three faint bands were smaller. This suggested that at least two of the Eco RI ras fragments are non-contiguous, and hence two to three ras genes may be present in addition to the one characterized by Reymond et al. (1984, Cell 39;141). All Northern and Southern bolts were probed with antisense RNA probes in order to gain greater sensitivity of detection as described by Cox et al. (1984, Dev. Biol. 101;485).
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LIST OF ABBREVIATIONS

cAMP  cyclic adenosine 3',5' monophosphate

_c-Ha-ras_ cellular homolog of the viral Harvey ras gene

c-Ki-ras  cellular homolog of the viral Kirsten ras gene

DAG  1,2-diacylglycerol

Dd-rascl  cDNA clone of Dictyostelium ras RNA isolated by Reymond et al. (1984, Cell 39, 141)

EGF  Epidermal Growth Factor

GDP  guanosine diphosphate

GTP  guanosine triphosphate

GTPase  guanosine triphosphatase

HMSV  Harvey Murine Sarcoma Virus

IP$_3$  inositol-1,4,5-phosphate

kd  kilodaltons

LTR  Long terminal repeat (of retrovirus genome)

mRNA  messenger RNA

MLV  Murine Leukemia Virus

MSV  Murine Sarcoma Virus

MTV  Murine Tumor Virus

NRK  Normal Rat Kidney

PIP$_2$  phosphatidylinositol-4,5-biphosphate

poly A+ RNA  polyadenylated RNA

RaR Ig  rabbit anti rat immunoglobulin G

rRNA  ribosomal RNA

S phase  DNA synthesis phase of cell cycle

SSV  Simian Sarcoma Virus

tRNA  transfer RNA
I wish to express my gratitude to my supervisors Dr. Tony Pawson and Dr. Gerry Weeks for providing me with the opportunity to do this work. I also wish to thank my parents and Ralph McLean for their support.
The conservation of oncogenes in the genomes of species ranging from vertebrates to the simplest eucaryotes suggested they played an important cellular role (Hunter, 1984). The transforming and tumorigenic effects of the viral oncogenes, and the latent potential of their cellular protooncogene homologs for "activation" to oncogenic forms suggested these genes controlled cell growth. Indeed, several oncogenes have now been recognized as analogs of growth factors (Waterfield et al., 1983; Doolittle et al., 1983), growth factor receptors (Downward et al., 1984; Sherr et al., 1985) or belonging to a family of tyrosine kinases similar to the EGF receptor (Hunter, 1984).

The ras members of the oncogene family were discovered as sequences carried by the Harvey and Kirsten murine sarcoma viruses that were responsible for tumorigenesis (Ellis et al. 1980, 1981). The ras gene has been of particular interest in cancer research as its activation may be responsible for 10 to 30% of all human malignancies (Lacal and Aaronson, 1986a; Finkel et al., 1984). An analog of the mammalian ras gene was found in the genome of the cellular slime mold Dictyostelium discoideum (Reymond et al., 1984). This organism has a simple developmental program that causes a population of undifferentiated single-celled amoebae to aggregate upon depletion of nutrients, and form a multicellular organism with two differentiated cell types. The cells undergo two periods of mitosis and DNA synthesis during development (Zada-Hames and Ashworth, 1978). Since there is some evidence to suggest
that ras function is important in the initiation of the S phase of the cell cycle (Feramisco et al., 1984; Mulcahy et al., 1985), and since in Dictyostelium there is some evidence that cell fate may be linked to the cell cycle (Weijer et al., 1984a; Sharpe et al., 1984) this organism is an interesting yet simple model in which to investigate a possible linkage of ras expression to the cell cycle as well as to differentiation. Indeed, ras has been already been found to be differentially expressed in D.discoideum, both temporally, and with respect to the two cell types (Pawson et al., 1985; Reymond et al., 1984). D.discoideum is amenable to transformation techniques (Barclay et al., 1983; Reymond et al., 1985) but does not have the advantage of site-directed transformation by gene replacement, as in yeast (Rothstein, 1983).

The viral and mammalian ras genes encode 21 kd proteins (p21 ras), known to bind GTP and GDP with high affinity (Shih et al., 1980), and to have low GTPase activity (McGrath et al, 1984; Sweet et al., 1984). Both the Harvey and Kirsten murine sarcoma virus (MSV) ras proteins have threonine in place of the normal alanine at position 59, and autophosphorylate at that site (Papageorge et al., 1982). The ras protein undergoes several post-translational changes. Harvey MSV p21 ras is derived from a larger precursor (Shih et al., 1982). Palmitic acid is covalently attached (Buss and Sefton, 1986) near the carboxyl terminus, most likely to cysteines at positions 184 or 185 (Willumsen et al., 1984) and is required to bind the ras protein to the inner surface of the plasma membrane. Transformation by activated forms of p21 ras will occur
only if the protein is in association with the plasma membrane. Proteins lacking amino acids 184 to 189 have no lipid attached, remain in the cytoplasm, and fail to transform NIH-3T3 cells (Willumsen et al., 1984). Since a portion of Harvey MSV p21 protein is autophosphorylated, and a portion of each of those species is fatty acylated, Buss et al. (1986) were able to resolve four distinct forms of HMSV ras protein in addition to the precursor.

The ras protein is striking in that the normal cellular form of the protein can be "activated" to its oncogenic form by single point mutations at position 12 (Tabin et al., 1982; Reddy et al., 1982; Capon et al., 1983; Shimizu et al., 1983) that cause a reduction in GTPase activity (McGrath et al., 1984), or at position 61 (Yuasa et al., 1983) that is likely part of the GTP binding site. There are 3 amino acid differences between the viral and cellular Harvey ras proteins, and 7 between the viral and cellular Kirsten ras proteins (Shimizu et al., 1983), that in each virus include a position 12 mutation and the substitution of threonine for the native alanine at position 59. Either change alone is sufficient to cause oncogenic activation (Lacal et al., 1986b). The effect of threonine at position 59 appears to be negated by the in vitro mutagenesis of asparagine 116 to either lysine or tyrosine, as this second mutation abolished guanine nucleotide binding, autophosphorylation, and transformation of cells (Clanton et al., 1986). Additional single amino acid changes sufficient to cause oncogenic activation were identified at positions 13 and 63 by mutagenesis (Fasano et al., 1984).
The ras sequences are so conserved between species that ras proteins from mammalian cells will function in yeast cells (Kataoka et al., 1985). Transfection of growing mouse NIH-3T3 or normal rat kidney (NRK) cells with an activated human Harvey ras gene produces foci of transformed cells, whereas the normal human Harvey ras gene produces little or no effect. However, both the normal murine and the normal human Harvey ras genes could transform NIH-3T3 mouse cells if transcribed in very high levels from a highly active viral LTR promoter (DeFeo et al., 1981, Chang et al., 1982).

Unexpectedly, quiescent mouse NIH 3T3 cells, quiescent normal rat kidney cells and rat embryo fibroblasts (REF-52) all underwent morphological changes and initiated DNA synthesis in depleted media, after the microinjection of the oncogenic form of the human Harvey ras protein (Feramisco et al., 1984). Feramisco et al. found that microinjection of the same levels (10^5 to 10^6 molecules per cell) of the normal human Harvey ras protein had little effect. However, Stacey and Kung (1984) reported some foci showing less pronounced transformation upon microinjection of high levels (≈6x10^7 molecules per cell) of the normal human Harvey ras protein. This result was consistent with the view of DeFeo et al. (1981) that elevated levels of the normal ras protein could lead to transformation. These findings suggest that the ras protein provides a signal to cells to enter the S phase of the cell cycle. Transformation was inhibited by both cycloheximide and actinomycin D, indicating a requirement for protein synthesis and transcription to mediate the transforming effects of ras (Feramisco et
It should be noted that not all cell lines respond to p21 ras injection (Feramisco et al., 1984; Stacey and Kung, 1984) or transfection by ras DNA (Sager et al., 1983). Cell lines such as NIH-3T3, which are highly amenable to transformation by ras via transfection or microinjection, may be in a penultimate stage of transformation or have cell-type specific properties that make them susceptible to ras stimulation (Land et al., 1983).

As an extension of prior experimentation with microinjection of ras proteins, Mulcahy et al. (1985) microinjected the anti-ras monoclonal antibody Y13-259 into quiescent NIH-3T3 cells prior to the addition of 10% fetal calf serum, and found that the antibody (used at about 3x10^6 molecules/cell, the equivalent of 0.2% of cell protein) abolished the DNA synthesis which would otherwise occur about 16 hours after the addition of fresh serum. DNA synthesis was not prevented by microinjection of either an anti-human interferon antibody or the monoclonal antibody Y13-238. This latter antibody is known to bind Harvey ras but not Kirsten ras proteins. That Y13-238 serves as a negative control in this experiment suggests that the endogenous cellular Kirsten ras (c-Ki-ras) protein was able to function in place of the c-Ha-ras protein, whereas binding of both c-Ha-ras and c-Ki-ras proteins by Y13-239 prevents transduction of a signal for DNA synthesis. This work adds to the evidence that the ras protein is required by cells to enter the S phase of the cell cycle.

Recently, it was demonstrated that pre-binding of Y13-259 antibody to
bacterially produced Ha-ras and Ki-ras proteins did not affect GTP binding, GTPase activity, or autophosphorylation (Lacal and Aaronson, 1986b). This implied that since Y13-259 binding does not interfere with these biological functions, it may neutralize ras function in vivo by binding to a site on the protein otherwise used to interact with another factor involved in signal transduction. Lacal and Aaronson (1986b) also used 3' deletions to localize the Y13-259 binding site to ras amino acids 69-89. Using this information, Papageorge et al. (1986) made mutant ras proteins lacking amino acids 64-72, 69-72, 72-76, and 72 alone, that escaped immunoprecipitation by Y13-259 and yet retained transforming ability. It was thought that methionine-72 was the critical component in the Y13-259 epitope. If the interaction site proposed by Lacal and Aaronson (1986b) exists, it can not require the region from amino acids 64 to 76.

Recent work by Papageorge et al. (1986) showed that substitution of the normal position 12 glycine had no effect on the GTP binding associated with the region around 59-61, and the substitution of threonine for alanine at 59 had no effect on the GTPase activity associated with position 12. Transfection of NIH-3T3 cells with ras DNA encoding either glycine 12-threonine 59 or lysine 12-alanine 59 produced foci with equal efficiency, demonstrating that either position 59 changes alone, or lower GTPase activity alone were sufficient to cause transformation. The threonine 59 mutation also increased GTP binding by 3 fold, although this increased binding did not affect GTPase activity in the assay performed.
Similarities between the ras proteins and the G proteins (the regulatory subunits of adenylate cyclase) led to a hypothesis that ras similarly acted on adenylate cyclase, to control intracellular cAMP levels (Sefton et al., 1982). The 45kd alpha stimulatory (G*s) and alpha inhibitory (G*i) G proteins reside in the membrane and have sites for ADP-ribosylation and GTP binding. When a stimulatory hormone binds its receptor, the receptor binds the G*s subunit, increasing the affinity of the latter for GTP. The entire complex binds adenylate cyclase which synthesizes cAMP until the hydrolysis of GTP by the G*s subunit terminates the stimulatory effect. The importance of this last step is demonstrated by the irreversible stimulation of the enzyme in the presence of non-hydrolysable GTP analogs (Gilman, 1984). Both the G and ras families of proteins are localized on the inner surface of the plasma membrane, both bind guanine nucleotides with high affinity (Scolnick et al., 1979), and both have weak GTPase activity (McGrath et al., 1984; Sweet et al., 1984). The ras proteins also have a small degree of amino acid sequence homology with the bovine Gi subunit (Hurley et al., 1984).

The discovery that position 12 activated forms of the ras protein had a 7 or 8 fold reduced GTPase activity relative to wild type (McGrath et al., 1984, and Sweet et al., 1984) led to an attractive model of oncogenesis, wherein ras activation would result in elevated levels of cAMP. This would overstimulate cAMP-dependant protein kinase, thought to be the only mediator of cAMP signals in eucaryotic cells. One of the regulatory subunits of cAMP-dependent protein kinase, RII, was found to possess topoisomerase I activity (Constantinou et al., 1985), which in
turn appeared to induce transcriptional activity in *Drosophila* polytene chromosomes (Fleishman et al., 1984), suggesting one way in which cAMP signals may be transduced to the nucleus.

This model appeared to be born out by the work of Toda et al. (1985) with *Saccaromyces cerevisiae*, an organism possessing two ras gene homologs. The levels of cAMP synthesized in the yeast reflected the number of functional ras genes, such that yeast lacking one ras gene, especially RAS2 showed low levels of cAMP, yeast with two ras genes showed normal levels of cAMP, and yeast that had their normal RAS2 gene replaced with a valine 19 activated ras gene (equivalent to the viral valine 12 mutation) showed highly elevated levels of cAMP. Yeast lacking both RAS1 and RAS2 were non-viable unless rescued by the bcyl ("bypass cAMP") mutation which results in a cAMP-independent protein kinase. These "ras1^-ras2^-bcyl" cells had low levels of cAMP, but were viable. Finally, yeast with only one RAS valine 19 gene were viable, but were unable to prepare properly for sporulation and failed to accumulate the carbohydrates associated with this process. This implied that cAMP levels must decline during the signalling to end vegetative growth and prepare for sporulation.

The requirement of ras genes for cAMP production and viability in yeast is not universal. Low stringency hybridizations of DNA from another species of yeast, *Schizosaccharomyces pombe*, indicated the existence of only one ras gene. When this site was disrupted by replacing the ras1 gene with selectable leu1 or ura4 markers, these yeast produced equal numbers of ras1- and ras1+ spores, all of which were
viable. Furthermore, levels of intracellular cAMP detected in both ras1- and ras1+ yeast were normal. But ras1- haploid yeast could not mate, and ras1-/ras1- diploids could not sporulate efficiently (Fukui et al., 1986). Unless a second ras gene has gone undetected in this species it would appear that ras is not universally required for viability in yeast, although its requirement in spore formation is implicated in both strains.

The work of Beckner et al. (1985) provided evidence that the ras protein was not a regulatory component of adenylate cyclase in mammalian cells, because it could not "rescue" a G•s- mutation in the human lymphoma line S49, which lacks the stimulating regulatory subunit of adenylate cyclase and requires exogenous cAMP for viability. cAMP could be synthesized in vitro utilizing adenylate cyclase activity from solubilized S49 membranes, and G•s activity from normal cell membranes. The latter had their endogenous adenylate cyclase activity abolished during an incubation. S49 membranes alone, or S49 membranes with bacterial or viral p21 (shown to retain GTP binding, GTPase activity and autophosphorylating activity in vitro) could not synthesize cAMP in this assay. There was also speculation that the p21 protein might serve as the G•i subunit, because cAMP levels are often below normal levels in epithelial and fibroblast cells lines transformed with Harvey or Kirsten ras. Yet bacterially-produced ras protein did not decrease the rate of cAMP synthesis in solubilized membrane assays of either normal or Harvey HSV transformed cells.

Further convincing evidence that ras does not interact with adenylate
cyclase in cells other than yeast, comes from work done with *Xenopus* oocytes. Oocytes removed from *Xenopus* ovaries are arrested in the prophase of meiosis, and agents which lower cAMP levels, such as insulin or the physiological inducer progesterone, will induce meiosis. Agents which increase cAMP levels, such as cholera toxin, phosphodiesterase inhibitors, or cAMP-dependent protein kinase inhibit maturation. Birchmeier et al. (1985) found that microinjected ras protein stimulated maturation, and furthermore, that valine 12 mutants (which would increase cAMP levels according to the model of Toda et al., 1985) were 100 fold more potent in bringing about meiosis. This suggested that the valine 12 ras protein must not be causing an increase in cAMP levels, indeed; no increase or decrease was detectable. Hence, in *Xenopus*, ras doesn't appear to interact with either adenylate cyclase or cAMP.

The cellular effects of ras activity outlined above will doubtless be re-examined in light of recent evidence that ras couples a second group of hormones that includes bombesin, bradykinin, and gastrin releasing peptide (GRP) to phospholipase C (Fleischman et al., 1986). This class of hormones act not via adenylate cyclase and cAMP, but via phospholipase C, which hydrolyzes phosphatidylinositol-4,5-biphosphate (PIP$_2$), a lipid component of the cell membrane into two breakdown products, both of which appear to have regulatory activity. Inositol-1,4,5-triphosphate (IP$_3$) causes the release of Ca$^{2+}$ into the cytosol from intracellular stores (Berridge and Irvine, 1984; Streb et al., 1983). 1-,2-diacylglycerol (DAG) activates protein kinase C (Bell et al., 1979; Nishizuka, 1984). Both Ca$^{2+}$ release and protein kinase C activate a plasma membrane Na$^+-$H$^+$
exchanger (Burns and Rosengurt, 1983; Moolenaar et al., 1984). This results in an elevation of cytoplasmic pH and Na+ levels, critical events in the growth factor stimulation of cell proliferation (Habenicht et al., 1981; Berridge et al., 1984). GTP is essential for the phospholipase C activity required to precipitate these events (Cockcroft and Gomperts, 1985). Fleishman et al. (1986) found that the levels of the breakdown product DAG to its precursor PIP$_2$ were 2.5 to 3-fold higher in NIH 3T3 and NRK cells transformed with viral and position 12 activated ras genes. In a more elegant experiment, Wakelam et al. (1986) transformed NIH 3T3 cells with the normal human N-ras gene under the control of the dexamethasone inducible MMTV promoter. Dramatic increases in IP$_3$ levels were seen in cells treated with both dexamethasone and those growth factors which act via phospholipase C activation, in particular bombesin, and gastrin releasing protein and bradykinin. Neither dexamethasone nor growth factors alone produced notable increases IP$_3$ pools, showing a requirement for coupling between ras and the growth factors involved, in order for PIP$_2$ to be hydrolyzed to IP$_3$.

Hence, ras may serve as the intermediary between a class of growth factors and phospholipase C, to allow cells to enter the S phase of the cell cycle. Activating mutations of the ras gene may encode ras proteins that act on phospholipase C independent of stimulation by growth factors.

The properties of a given cell line may also influence the effects of ras gene activation. Cells of the human breast cancer cell line MCF-7, for example, produce tumors in nude mice and in ovariectomized nude mice given estrogen, showing a requirement for estrogen for tumorigenicity.
Transfection of MCF-7 cells with the activated viral Harvey ras gene produces cells that form tumors in ovariectomized mice in the absence of estrogen (Kasid et al., 1985). This effect contrasts with the ras-stimulated initiation of mitosis in quiescent NIH 3T3 fibroblasts, and of meiosis in Xenopus oocytes. In all three examples, a normal requirement for growth factors can be abolished by a ras gene expressed in elevated levels, or in an activated form.

Dictyostelium is an interesting model in which to study the connection between ras expression and cell division because mitosis occurs in two distinct periods during development (Zada-Hames and Ashworth, 1978), and because cell fate may be linked to the cell cycle (Feramisco et al., 1984; Mulcahy et al., 1985). The first period of mitosis peaks at 4 hours of development (beginning at 0 hours and lasting until the first signs of rippling at 8-9 hours), the second period of mitosis starts with a pronounced peak at first finger stage which declines towards culmination (Zada-Hames and Ashworth, 1978). D. discoideum has been of interest to biologists primarily for its ability to exist either as a population of single cells, or as a multicellular organism displaying two differentiated cell types. Within hours after the food supply has been depleted, amoebae aggregate to form mounds containing about 10 cells each. By 14 hours of development the aggregated cells form a migrating slug, with cells which are partially differentiated into the stalk cells of the mature structure (prestalk cells) localized at the anterior of the slug, and the "prespore" cells localized at the posterior end. By 24 hours of development the prespore cells are borne aloft by the upwardly
elongating stalk, to form the mature fruiting body.

Cyclic AMP in *D. discoideum* functions in chemotaxis, in the transcription of post-aggregation dependant mRNA species (Chung et al., 1981), and in the maintenance of expression of many developmental genes (Barklis and Lodish, 1983). About 6 hours after the depletion of nutrients, amoebae begin to stream towards individual cells in the population which emit periodic pulses of cAMP. The cells produce an extracellular and a cell surface phosphodiesterase which break down extracellular cAMP. The balance of these factors creates pulsatile signals and a chemotactic gradient, emanating from the center of each aggregation territory. The expression on the cell surface of both cAMP binding protein and cAMP phosphodiesterase remain low until 6 hours of development, rise to sharp peaks when aggregation is complete at 8 hours, and then decline towards culmination (Henderson, 1975). This reflects the requirement of the cAMP signalling system to establish aggregates. The secretion of cAMP itself rises steadily from 9 hours, peaks at 15 hours, and then declines sharply (Tyler and Bonner, 1969). This may reflect a lower requirement for cAMP to maintain the differentiated state of prestalk and prespore cells as they approach terminal differentiation.

In rapidly shaking cultures, where cell-cell adhesion is inhibited, cAMP alone appears sufficient to induce the transcription of prestalk-specific genes, whereas both cAMP and cell-cell contact are required to induce the transcription of prespore-specific genes (Mehdy et al., 1983). Another factor identified as necessary for cell-specific gene activation is DIF (differentiation inducing factor), a dialyzable, lipid-like factor.
which seems to be required for the formation of stalk cells (Kopachik et al., 1983).

The disaggregation of partially developed cell masses causes a rapid loss of cell-type specific gene transcription, and a reduction of some aspects of gross cell morphology such as prestalk and prespore vacuoles. When disaggregated cells are prevented from reaggregating in fast shake-suspension cultures, the addition of exogenous cAMP restores partially or completely the transcription of all cell-type specific genes. Recovery of expression of cell-type specific RNAs was not seen in cultures that did not receive exogenous cAMP (Barklis et al., 1983). Chung et al. (1981) derived a half-life of 4 hours for both vegetative and aggregation-dependant mRNA in pseudoplasmodial stage cells. When pseudoplasmodial stage cells were disaggregated, the half-life of only the aggregation-dependant mRNAs was reduced to 25 to 45 minutes. Possibly, RNase activity is important in ending the expression of aggregation-dependant mRNA in disaggregated cells.

Many differences are apparent in the role of cAMP in Saccharomyces cerevisiae and in D.discoideum. In S.cerevisiae, low cAMP levels appear to signal both the depletion of nutrients and the preparation for sporulation. The expression of a position 12 activated ras gene in S.cerevisiae appeared to interfere with the preparation for sporulation by causing constitutively high levels of cAMP (Noda et al., 1985). Conversely, in D.discoideum the cellular response to depletion of nutrients is a prolonged period of elevated cAMP synthesis. Furthermore, elevated cAMP levels are required to initiate and maintain the
differentiated state of prespore cells. Hence, if ras acts on adenylate cyclase in Dictyostelium, it does so in a manner vastly different to that in S.cerevisiae.

A Dictyostelium discoideum ras gene originally isolated by Reymond et al. (1984) as a prestalk-specific cDNA, was found to have 64 to 66% protein homology with the ras genes of other species. A probe made from the D.discoideum ras cDNA hybridized to 0.9 and 1.2 kb transcripts in strain AX-3 RNA. Both mRNAs were strongly expressed in the poly A+ RNA of AX-3 cells at 15 and 17.5 hours of development. Levels of the two transcripts declined steadily to zero in the RNA of cells at 20, 22.5 and 25 hours of development. Prior to 15 hours of development, the only detection of a ras mRNA was in RNA from vegetative cells, and only the 1.2 kb transcript was present. A part of the D.discoideum ras gene, encoding amino acids 59 to 187, was cloned into a gtll expression vector, and the resultant E.coli -galactosidase/ras fusion protein was used to produce a polyclonal anti-ras serum in rabbits. When Western blots of protein from D.discoideum cells at various stages of development were probed with this immune serum, the greatest amount of ras protein appeared in vegetative cells, and levels declined steadily to zero at 25 hours. This pattern of expression was in striking contrast to that seen in Northern blots, and Reymond et al. (1984) suggested that the ras mRNA may be more labile than the protein.

In contrast, Pawson et al. (1985) analyzed the expression of in vivo pulse-labelled ras proteins in strain V12M2. The monoclonal antibody Y13-259 immunoprecipitated two Dictyostelium ras proteins of 23 and 24
kd. The 24 kd protein was a minor species not always detected, but was expressed predominantly in pseudoplasmodial stage cells when seen. High total levels of ras protein were detected in vegetative cells, followed by a marked increase in synthesis in the first one to two hours of development. A second smaller burst of expression occurred during pseudoplasmodial formation (at 12 hours of development), after which ras protein levels diminished towards culmination. The 23 and 24 kd Dictyostelium ras proteins appeared closely related to each other as well as to the Harvey viral ras protein, as determined by tryptic peptide analysis.

Both the Northern blots of Reymond et al. (1984) (using strain AX-3) and the immunoprecipitations of Pawson et al. (1985) (using strain V12M2) showed two peaks of ras expression, one at 0 to 2 hours, and the other at the pseudoplasmodial stage. The most obvious difference between the results of the two labs was that more ras protein was immunoprecipitated in strain V12M2 vegetative cells relative to pseudoplasmodial cells (Pawson et al., 1985) whereas far more mRNA was detected in strain AX-3 pseudoplasmodial cells relative to vegetative cells. Possible explanations for the lack of correlation between the levels of ras protein and ras mRNA are firstly, strain differences; secondly, dramatic changes in the efficiency of translation of ras mRNA throughout development; and thirdly, the presence of a second ras gene not detected in the Northern blots of Reymond et al. (1984), which is actively transcribed in vegetative and early developing cells. In support of this last theory is the finding of Weeks and Pawson (1986) that the ras
protein is located predominantly in prespore cells, whereas Reymond et al. have found the ras mRNA to be predominantly in prestalk cells. It is noteworthy that all other organisms thus far studied with the exception of Schizosaccharomyces pombe contain more than one ras gene. It was decided to examine ras expression further by applying both protein and RNA methodologies to a single strain of D. discoideum, V12M2. Levels of ras protein were examined by immunoprecipitating proteins translated in vitro from the RNA used in Northern blots. Low stringency Southern blots were also used to search for the putative second ras gene. Antisense RNA probes were used instead of nick-translated DNA probes, in the hope that the greater sensitivity of the SP6 RNA probe system would allow the detection of heterologous ras mRNA or genomic loci.

My results show that the highest levels of ras protein are translated in vitro from vegetative and 2 hour RNA, but that the highest levels of ras mRNA are present in the RNA of cells at the pseudoplasmodial stage of development. Hence the level of ras protein synthesized in vitro did not correspond to the levels of ras RNA detected at different stages during development. The kinetics of the in vitro synthesis of the two Dictyostelium ras proteins suggest that they are not derived from one another and hence are translated from two different mRNAs. It is not known whether these two mRNAs are derived from the same ras gene or not. Novel genomic restriction fragments containing ras-homologous sequences were detected by use of an antisense RNA probe under conditions of low stringency.
Growth and differentiation of D.discoideum strain V12M2. Amoebae were grown in suspension cultures on E.coli B23 which had been resuspended from M9 media to an optical density at 660 nm of 6 OD in sterile KK2 buffer (20 mM potassium phosphate monobasic/potassium phosphate dibasic, pH 6.0). Amoebae were generally inoculated at a concentration of 2x10^5 cells/ml, and harvested before they had reached a concentration of 6x10^6 cells/ml. To induce differentiation, amoebae were separated from bacteria by 3 to 4 differential centrifugations (700g for 2 min), in KK2 buffer. Amoebae were counted with a haemocytometer, and 10^8 cells were pipetted onto Millipore filters resting on Millipore pads saturated with 0.8 ml KK2 buffer containing 0.5 mg/ml streptomycin. In vivo labelling was carried out by transferring filters onto a droplet of 250μCi [35S]-methionine for a two hour period, followed by three washes of the cells in KK2 buffer to remove exogenous label.

Extraction of RNA. RNA was extracted using the methods of Blumberg and Lodish (1980). Cells at the appropriate stage of development were washed from filters with KK2 buffer and pelleted. Each 2x10^8 cells was vortexed with 4 ml of lysis buffer containing 50 mM Hepes, 40 mM magnesium acetate, 20 mM potassium chloride, and 0.2% sodium dodecyl sulfate (SDS). This aqueous phase was immediately vortexed with an equal volume of water-saturated phenol. After adjustment to 0.2 M sodium acetate, 4 ml
of chloroform was added (per 2x10⁸ cells), and vortexed. The mixture was centrifuged for 10 minutes at 10,000 rpm, and the upper, aqueous layer transferred to a fresh tube. Two more phenol-chloroform extractions were carried out. The aqueous layer was then extracted twice with two 1.5x volumes of chloroform. Finally, the aqueous layer was brought to 0.3 M sodium acetate, 2 volumes of 95% ethanol was added, and the RNA was precipitated at -20°C. Precipitation was often carried out overnight, but 1 hour was sufficient to precipitate milligram quantities of RNA. The RNA was then pelleted 20 minutes at 10,000 rpm, and washed twice with volumes of 75% ethanol equal to the original precipitation volume. The pellet was vacuum-dried, resuspended in sterile distilled water, and ethanol precipitated a second time. The RNA was pelleted a second time, washed twice in 75% ethanol, vacuum-dried, and resuspended to 2.5 mgs/ml in sterile distilled water. Generally, the RNA yield from early developing cells was 0.9 to 1 mg per 10⁸ cells, and decreased to 0.5 mg per 10⁸ cells by 15 hours. Nitric acid-cleaned Corex tubes were used throughout.

Subcloning ras probes into SP6. Antisense RNA probes were used instead of nick-translated DNA probes, so that the greater sensitivity of RNA probes might allow [in contrast to DNA probes (Reymond et al., 1984)] the detection of heterologous ras mRNA and genomic sequences. Cox et al. (1984) established that the association and disassociation temperatures of RNA duplexes were 20°C higher than the corresponding temperatures for
DNA duplexes (55C and 75C respectively, for RNA, compared to 35C and 55C for DNA in 50% formamide). Hybridization and washes can be carried out at higher temperatures, and only the antisense strand of the probe becomes labelled by SP6 polymerase, producing a high signal to noise ratio. In formamide concentrations over 40%, RNA:DNA duplexes are also more stable than DNA duplexes, especially as G+C content increases (Casey and Davidson, 1977). Of interest is the finding of Cox et al. (1884) that two complementary histone RNAs that differed by 10-15% in their RNA sequences hybridized at 42C, or 13C below the temperature of duplex formation of + and - RNA strands.

**Constructs:**

i) The 470 bp PstI fragment of a *D.discoideum* ras cDNA clone called "Ddrascl", kindly provided by Richard Firtel, was excised from pBR322 and ligated into PstI-digested SP64. Both orientations were recovered. SP6 polymerase transcribes from the antisense orientation, 94 nucleotides of 3' ras gene flanking sequence, followed by the coding sequence corresponding to amino acids 186 to 59.

ii) A Harvey murine sarcoma virus (HMSV) ras probe was made by ligating the 700 bp PstI-HindIII HMSV ras gene fragment from plasmid "14" (Ellis et al., 1980) into the SP65 vector which had been digested with the same enzymes. SP6 polymerase transcribes approximately 60 nucleotides of viral sequence 3' of the HMSV ras gene, followed by the HMSV ras coding sequence corresponding to amino acids 189 to 1.

iii) A Kirsten murine sarcoma virus (KMSV) ras probe was made by
ligating the 1.2 kb BamHI-XbaI KMSV ras gene fragment from the plasmid "KBE-2" (Ellis et al., 1981) into the SP64 vector linearized with the same enzymes. SP6 polymerase would transcribe the KMSV ras coding sequence corresponding to amino acids 120 to 1, followed by 420 bp of viral ras 5' flanking sequence.

Homology of the three probes to one another (as determined by use of the Delaney SEQUENCE program):

<table>
<thead>
<tr>
<th></th>
<th>Harvey MSV ras</th>
<th>Kirsten MSV ras</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dd-rascl</td>
<td>53% overall</td>
<td>61% overall</td>
</tr>
<tr>
<td></td>
<td>69% in the first 100</td>
<td>75% in the first 100</td>
</tr>
<tr>
<td></td>
<td>bp of coding region</td>
<td>bp of coding region</td>
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<tr>
<td>Kirsten MSV ras</td>
<td>60% overall</td>
<td></td>
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<tr>
<td></td>
<td>82% in the first 100</td>
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<tr>
<td></td>
<td>bp of coding region</td>
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Preparation of radioactively labeled probes. Radioactive probes were prepared as described in the Bio-Can Riboprobe instruction leaflet. 20 µl transcription reactions contained 1.6 µg of linearized template, 12 µM
radioactively labelled  \( ^{32}P \)-CTP (600 Ci/mmole), 1x transcription buffer [200 mM Tris-chloride (pH 7.5), 30 mM magnesium chloride, 10 mM spermidine, and 100 mM sodium chloride], 10 mM DDT, 20 units RNAsin inhibitor, 0.5 mM each of ATP, GTP, and UTP; and 13 units SP6 polymerase (8 units per \( \mu \)g template). Transcription was carried out at 40°C for 1 hour. The DNA template was then removed by incubation at 37°C for 15 minutes with 0.4 \( \mu \)g DNase and 0.6 \( \mu \)l RNAsin inhibitor. Proteins were removed by one extraction with an equal volume of water-saturated phenol and one extraction with an equal volume of chloroform. The aqueous layer was brought to 2.0 M ammonium acetate, and then 10 \( \mu \)g of carrier tRNA and two volumes of ethanol were added. 4 hours at -80°C, or overnight at -20°C provided adequate time for precipitation. Probe was pelleted at 10,000 rpm for 45 minutes, and washed once with 70% ethanol. After the probe was dried in a spin-vacuum apparatus and resuspended in 100 \( \mu \)l of sterile water, 1 \( \mu \)l was removed for Cerenkov counting. The probe was used within a few days of synthesis.

**Northern blots.** 20 \( \mu \)g of total RNA or 3 to 6 \( \mu \)g of poly A+ RNA were dried in Eppendorf tubes and resuspended in 5.3 \( \mu \)l 30 mM sodium phosphate monobasic/sodium phosphate dibasic (pH 7.0), and 8 \( \mu \)l dimethylsulfoxide. 2.7 \( \mu \)l of deionized 40% glyoxal was added to each tube and samples were incubated 1 hour at 50°C. 3 \( \mu \)l of loading dye [50% glycerol, 10 mM sodium phosphate monobasic/sodium phosphate dibasic (pH 7.0), 0.4% bromophenol blue] was added to each RNA immediately prior to
electrophoresis. The gel bed and box were soaked in 25 mM iodoacetate for 30-60 minutes, during the preparation of the gel [1.1 or 1.2% agarose in 10 mM sodium phosphate monobasic/sodium phosphate dibasic (pH 7.0), containing 0.5% (w/v) iodoacetate]. The buffer was circulated during electrophoresis with a peristaltic pump. Gels were run at 90 V, after which lanes containing rRNA size markers were excised, soaked 30 minutes in 50 mM sodium hydroxide in order to remove the glyoxal, then soaked 30 minutes in 10 µM ethidium bromide, and photographed. The gel itself was blotted overnight onto nitrocellulose, which was then baked in vacuo for 2 hours at 80 C.

Northern hybridization was carried out as per Melton et al., 1984. Prehybridization was carried out for one to four hours in the following: 50% formamide (deionized overnight on Bio-Rad AG 501-X8 resin and stored at -20C), 50 mM sodium phosphate monobasic/sodium phosphate dibasic (pH 6.5), 5x standard salt citrate, SSC (20x SSC is 3.0 M sodium chloride and 0.3 M sodium citrate, pH 6.8), 0.1% SDS, 1 mM EDTA, 200 µg/ml denatured salmon sperm DNA, and 0.05% Denhardt's reagent [1% Denhardt's reagent is 1% (w/v) each of bovine serum albumin, Ficoll, and polyvinlypyrolidine]. Hybridization was carried out for a minimum of 16 hours in the same solution, with the addition of 4x10^5 cpm of radioactively labeled RNA probe per cm^2. Three 20 minute washes were carried out in 0.1% SDS, 0.1x SSC. Stringent hybridizations were carried out at 55C, and washed at 65C. Low stringency hybridizations were carried out at 40C, and washed at 40C.
Growth of *D. discoideum* strain AX-3 and extraction of genomic DNA. Cells were grown axenically in shake suspension at 22°C in HL-5 media [1.5% w/v Bacto-peptone and yeast extract, 4.3 mM potassium phosphate monobasic/potassium phosphate dibasic (pH 7.0), 4.3 mM sodium phosphate dibasic] to a density of 5x10^6 cells/ml, and DNA extracted according to the methods of Daphne Blumberg (personal communication). 5x10^8 cells were washed 3 times in KK2 buffer, pelleted, and resuspended to 5x10^9 cells/ml, in the following lysis buffer: 10% sucrose, 0.5% NP40, 16 mM potassium chloride, 15 mM sodium chloride, 0.15 mM spermine, 0.5 mM spermidine, 15 mM β-mercaptoethanol, 15 mM Tris-chloride (pH 7.4), 1 mM EDTA, 0.2 mM EGTA, and 1.5 mM phenylmethylsulfonylfluoride. Cells were shaken vigorously in this buffer, pelleted at 4000 rpm for 10 minutes in a Sorvall GSA rotor, and the cloudy supernatant discarded. Nuclei were washed free of debris by two more washes in the same buffer, but without NP40. The nuclear pellet was resuspended in 15 ml sterile water, and adjusted to final concentrations of 0.1 M EDTA (pH 8) and 4% N-laurolsarcosine. Nuclei were lysed by gentle swirling at 50°C. Sigma type XIII protease was added to a final concentration of 200 μg/ml, and the mixture was incubated for 2 hours at 37°C with occasional swirling. The mixture was brought to 0.5 mg/ml ethidium bromide, and then cesium chloride was added to 0.905 gm/ml. This mixture was loaded into wide-mouth ultracentrifuge tubes, topped with parafin oil, and spun at 26,000 rpm for 36 hours in a SW28 rotor. The protease treatment was necessary to allow the DNA to escape the protein at the top of the tube. A small pellet of RNA and debris was
visible at the bottom of the tube. The banded DNA was withdrawn with a Pasteur pipette inserted through the protein layer. After multiple butanol extractions, the DNA was dialysed for 36 hours in several changes of 1x TE [10 mM Tris-chloride (pH 7.5) and 1 mM EDTA]. The DNA in solution was recovered from the dialysis tubing, and sodium acetate was added to give a final concentration of 0.25 M. DNA was then ethanol precipitated overnight. The OD 260/280 ratio of the DNA obtained by this method was 2:1, and the DNA appeared to digest well with the restriction enzymes used.

Southern blots. Hybridization of ras probes with digested genomic DNA from D.discoideum strain AX-3 was carried out as described in Southern protocol #4 provided in the Bio-Can Riboprobe leaflet. Prehybridization was carried out at 50C for 4 to 6 hours in 50% formamide, 5x SSC, 20 mM sodium phosphate monobasic/sodium phosphate dibasic (pH 7.0), 0.5% SDS, and 0.05% Denhardt's reagent. Hybridization was carried out at 50C for a minimum of 16 hours in the same solution, but with the addition of 250 ug/ml denatured salmon sperm DNA, and 4x10^5 cpm/cm^2 of radioactively labelled RNA probe. Two 30 minute washes were carried out at 55C in 2x SSC, then one wash at 55C in 0.5x SSC, followed by one or two 30 minute washes in 0.2x SSC. Low stringency hybridizations employed the methods of Madaule et al. (1984). Prehybridization was carried out for 8 hours at 42C in 30% formamide, 5x SSC, 5 mM EDTA, 0.1% SDS, 0.04% BSA, 0.04% Ficoll, 0.04% polyvinlypyrolidine, 12 mM sodium phosphate monobasic/
sodium phosphate dibasic (pH 7.0), 0.06% (w/v) pyrophosphate tetrasodium decahydrate and 100 μg/ml salmon sperm DNA. Hybridization was carried out at 42°C for 20 hours in 30% formamide, 5x SSC, 5 mM EDTA, 0.1% SDS, 0.04% Denhardt's reagent, 20 mM sodium phosphate monobasic/sodium phosphate dibasic (pH 7.0), 0.06% (w/v) pyrophosphate tetrasodium decahydrate, 50 μg/ml salmon sperm DNA, plus 4x10^5 cpm/cm² of radioactively labelled RNA probe. Washes were carried out at 42°C for 45 minutes each in 2x SSC, 2.5 mM EDTA, 0.1% SDS, 20 mM sodium phosphate monobasic/sodium phosphate dibasic (pH 7.0), and 0.06% (w/v) pyrophosphate tetrasodium decahydrate.

In vitro translation of D.discoideum RNA and immunoprecipitation of products. Messenger dependant rabbit reticulocyte lysate (MDL) was made according to the method of Jackson and Hunt (1982). MDL was mixed with [35S]-methionine (Amersham) to give a final concentration of 2 μCi/μl. 30 μl of this mixture was added to dry RNA which was resuspended, and translated in vitro for 2 hours at 30°C. Incorporation of methionine was optimal with 2.2 to 3.8 μg of total RNA/10 μl MDL, or with less than 0.4 μg of poly A+ RNA/10 μl MDL. After translation, 5 μl of lysate was added to 1 ml water and 0.5 ml 1 M sodium hydroxide/1.5% (w/v) peroxide was added. The mixture was incubated for 15 minutes at 37°C prior to the addition of 1 ml 20% trichloracetic acid (TCA). Precipitates were stored on ice for 20 minutes, and poured through 2.5 cm Whatman fiberglass GF/A filters, and washed four times with 2ml 5% cold TCA. The remaining 25 μl
lysate was diluted with 75 µl of 259 lysis buffer (0.5% SDS, 5 mM magnesium chloride, 100 or 400 mM sodium chloride, 20 mM Tris-chloride, 1% Triton X-100) plus 50 µl rabbit-anti-rat IgG-coated Staphylococcus ("Rarig Staph") resuspended to 10% (w/v) in the same buffer. After a 5 minute incubation at 4C, the mixture was centrifuged at 10,000 rpm for 2 minutes, and supernatants were transferred to fresh tubes containing 5 µg of Oncogene Sciences Y13-259 antibody. After overnight incubation on a tube rotator at 4C, 110 µl RaRIg was added to each tube for 2 hours and the mixture incubated for a further 2 hours. Finally, the immune complexes were centrifuged at 5000 rpm for 20 minutes, and washed 3x with 259 lysis buffer. The inclusion of 0.5% SDS in the 259 lysis buffer reduced the total yield of ras protein in immunoprecipitates, but was essential to reduce backgrounds to acceptable levels. On advise from Dr. Virginia Chow, RaRIg without staph was used in immunoprecipitations, and vastly improved immunoprecipitation backgrounds.
RESULTS

I) NORTHERN BLOTS OF DICTYOSTELIUM DEVELOPMENTAL TIME-COURSE RNAs.

The probe sequence, subcloned into the SP64 transcription vector, was a 470 bp Pst I fragment containing the coding sequence for amino acids 59 to 186 of the Dictyostelium ras protein. The fragment, kindly provided by Christophe Reymond, was part of the cDNA clone "Dd-rascl".

1) Measurement of level of total cellular ras RNA at different times during development.

Northern blots of total cellular D.discoideum RNA probed with Dd-rascl indicated that the 0.9 and 1.2 kb ras transcripts both appeared at 5 hours of development, and increased steadily until the last time point for which RNA was obtained, at 15 hours (Figure 1a). Beneath the ras RNA doublets in lanes 3 to 7 of Figure 1a, there was a tail of heterologously-sized ras transcripts ranging from 900 to 450 nucleotides long, decreasing in abundance with decreasing length. RNA degradation was the most likely cause of this tailing beneath bands. A longer exposure of this Northern blot revealed the presence of low levels of ras mRNA in the RNA from 2 hour cells, but no ras specific mRNA was detected in 20 ug of total RNA from vegetative cells (data not shown). (However, low levels of ras mRNA were detected in 6 ug of poly A+ vegetative RNA, data not shown). The low level of ras mRNA at 0 and 2 hours is not likely due to degradation by RNAses, since these early RNAs gave rise to abundant ras protein when in vitro translated. While it is possible that the ras RNA detected by the Dd-rascl probe does not encode the protein immunoprecipitated by the Y13-259 antibody, the fact that so much protein
Figure 1a. Northern blot showing the developmental expression of D.discoideum ras in total RNA from strain V12M2, probed with Dd-rascl RNA.

Total cellular RNA was phenol-chloroform extracted from developing cells at the times indicated. 20 µg of each RNA was glyoxalated and separated by electrophoresis in 10 mM sodium phosphate monobasic/sodium phosphate dibasic (pH 7.0), through a 1.1% agarose gel. RNA was then blot-transfered to nitrocellulose. Hybridization with the Dd-rascl probe was carried out in 50% formamide at 55C for 16 hours, followed by three 20 minute washes at 65C in 0.1x SSC, 0.1% SDS. Exposure was 2 days, with an intensifying screen.

Figure 1b. Northern blot with resolution of the two D.discoideum ras RNA species.

Method as above, with the inclusion of one 20 minute wash at 80C. Lane contains 20 µg of total RNA from cells at 8 hours of development.
is synthesized from the 0 and 2 hour RNAs suggests their general integrity is at least equal to that of RNA samples from later time points.

High stringency washes of Northern blots allowed resolution of the full length bands of the ras doublet (figure 1b). The sizes of the ras RNA bands detected in this work appeared to be the same as those reported by Reymond et al. (1984), determined in the following manner. It has been noted that RNA probes may bind non-specifically to 18S and 28S rRNA in low stringency Northern blots (Promega bulletin, 1986). In this work rRNA migration was first checked by ethidium bromide staining of one lane of each gel to be used in Northern blotting. When bands appropriately sized to be rRNA were detected in low stringency Northern blots, they were used as internal size markers. Assuming linear migration of RNA species, the sizes derived for the two ras transcripts were consistent with those reported by Reymond et al. (1984).

In summary, Northern analysis of total RNA from cells at different stages of development showed that the level of ras RNA in strain V12M2 increases steadily throughout the developmental program, rather than bimodally, as suggested by the level of ras protein immunoprecipitated from lysates of cells during development (Pawson et al., 1985) and by the level of ras RNA found in AX-3 cells during development (Raymond et al., 1985).

II) SOUTHERN BLOTS OF AX-3 DNA:

DNA from strain AX-3 was used because the sizes of the ras genomic fragments in this strain were known (Reymond et al., 1984). The use of
DNA from axenically grown cells also obviated artifacts seen in earlier Southern blots of DNA from bacterially grown V12M2 cells which possibly resulted from non-specific binding of the probe to bacterial sequences.

1) **High Stringency Southern Blots Using the Dictyostelium ras probe:**

The bands detected by probing genomic DNA under conditions of high stringency with the *Dictyostelium ras* probe appeared to be the same sizes as those found by Reymond et al. (1984). The *ras* gene mapped to a 5.3 kb Eco RI restriction fragment, a 3.2 kb Eco RI-Bgl II restriction fragment, and to a Eco RV restriction fragment approximately 11 kb long (figure 2). The different intensities of binding were probably due to the better transfer of the shorter pieces of DNA.

2) **Low Stringency Southern Blots Using the Dictyostelium ras probe:** The same blot was then re-probed with the *Dictyostelium ras* sequence under conditions of low stringency, at 42°C in 30% formamide (Madaule et al., 1985). Additional bands were indeed seen (figure 3). Their detection in this work, but not in low stringency Southern blots carried out by Reymond et al. (1984), may have been facilitated by the large amount of DNA used per lane (15 μg), or because of the greater sensitivity of the probe system used. The Eco RV digested DNA showed faint bands in addition to the dominant 11 kb band, of 6.1 and 13.5 kb (lane 3). In undigested DNA (lane 1) the *ras* probe localized to a region of the blot corresponding to about 15 kb. In addition to the dominant band of 5.3 kb in Eco RI digests, three other faint bands were seen of 3.3 kb, 7.0 kb, and of about 9.1 kb (lane 2). In addition to the dominant band of 3.2 kb in Eco RI-Bgl II digests, three faint bands were seen of 1.75 kb, 5.5 kb,
Figure 2. Southern blot of AX-3 DNA, probed at high stringency with Dd-rascl RNA.

Hybridizations with the Dd-rascl RNA probe were carried out in 50% formamide at 45°C for 18 hours, then washed for 20 minutes once at 50°C in 2x SSC, once at 50°C in 0.5x SSC; then twice at 50°C in 0.2x SSC. Exposure was 20 hours, without a screen. 15 μg of DNA, digested with the following restriction enzymes, was used per lane. Lane 1: undigested. Lane 2: Eco RI. Lane 3: Eco RV. Lane 4: Eco RI/Bgl II.
Figure 3. Southern blot of AX-3 DNA, probed at low stringency with Dd-rascl RNA.

As for figure 3. Hybridization was carried out in 30% formamide at 42°C, for a minimum of 18 hours. The blot was washed twice for 30 minutes at room temperature and twice for 30 minutes at 42°C, in the solution specified in Methods for low stringency Southern blots. Exposure was 16 hours with screen.
and of 9.1 kb (lane 4). Possibly the 9.1 Eco RI fragment was not further
digested by Bgl II, whereas the 7.0 and 3.3 kb Eco RI bands gave rise to
the 5.5 and 3.2 kb bands in the Eco RI-Bgl II digest by cleavage in 5' or
3' flanking regions. If so, this would suggest that the three Eco RI
bands represent at least two and possibly three non-contiguous ras-
homologous sequences separated by many kilobases, therefore indicating
the presence of two to three ras-homologous genes in addition to the one
characterized by Reymond et al. (1984).

The faint bands detected in Figure 3 are not likely artifacts caused
by partial digestion, firstly because two of the bands are smaller than
the dominant species seen under conditions of high stringency, and
secondly because partial digestion products present in large enough
quantities to be detected under conditions of low stringency, would be
similarly detectable under conditions of high stringency.

The optimum binding conditions for the high and low stringency bands
were not established. It was however noted that when the same DNA blot
used in Figures 2 and 3 was re-probed at 40°C instead of 45°C in 50%
formamide, the dominant bands seen in Figure 2 plus only three of eight
of the faint bands seen in Figure 3 were detected. These were the 7.0 kb
band of lane 2, Figure 3; the 13.5 kb band of lane 3, and the 5.5 kb band
of lane 4 (data not shown).

3) Southern Blots Of AX-3 DNA Using Viral ras Probes:

Neither the Kirsten nor the Harvey probe hybridized to the
Dictyostelium ras, or any other genomic sequence on a blot of digested
AX-3 DNA. At the lowest stringency used, 42C in 30% formamide; blots had
high, even backgrounds (data not shown). As noted in the Methods, the HMSV and KMSV ras coding sequences share 82% homology (as determined by use of the Delaney SEQNCE program) in the first 100 base pairs of the ras coding sequence (the most highly conserved region) and cross hybridize under conditions of low stringency (Chang et al., 1982). In contrast, the Harvey and Kirsten ras sequences share only 69% and 75% homology respectively with the Dictyostelium ras sequence in the first 100 base pairs of coding sequence, as determined by use of the Delaney SEQNCE program. The Dictyostelium and HMSV sequences share one stretch of 11 base pairs of unbroken sequence homology in this region, and the Dictyostelium and KMSV sequences share one unbroken stretch of 14 base pairs. Other stretches of unbroken sequence homology are much shorter.

In summary, new information from this work was primarily the detection by use of a D.discoideum ras probe at low stringency, of heterologous ras genomic sequences in D.discoideum strain V12M2 DNA. The results suggest the presence of two to three sequences heterologous to the probe sequence.

III) IMMUNOPRECIPITATION OF D.DISCOIDEUM RAS PROTEINS.

In view of the differences in developmental gene expression detected in the studies of Reymond et al. (1984) and Pawson et al. (1985), RNA prepared from cells at each developmental time point were translated in vitro and immunoprecipitated, firstly to confirm that ras protein could be translated in vitro from vegetative RNA despite the apparent absence of ras RNA in vegetative cells, and secondly to see if levels of ras protein were co-ordinate with the levels of ras RNA detected in Northern
blots. A number of intriguing and potentially important differences were found between the ras proteins synthesized in vivo and in vitro.

Comparison of in vitro translated ras proteins with in vivo synthesized ras proteins.

In cell-free lysates of strain V12M2, the dominant ras protein was 23 kd. p24 was a minor species not always seen in vivo, but when present was most abundant in pseudoplasmodial cells at 14 hours. Although the pulse-chase labelling experiments required to establish the relationship between the in vivo ras proteins were made difficult by the poor exchange between intracellular pools and exogenous methionine, particularly in older developing cells, there was no evidence to suggest that p24 was a precursor to p23 in vivo (Weeks and Pawson, 198).

In contrast, both p23 and p24 were synthesized in vitro from the RNA isolated from V12M2 cells at all time points from vegetative stage to 15 hours of development. Furthermore, the 24 kd ras species was translated in equal or greater amounts than was the 23 kd ras species from the RNA from cells at all time points except 15 hours, when p23 frequently became the predominant ras protein species (figure 4). The different ratios of p23 and p24 obtained in vivo and in vitro may be due to the expression of the Dictyostelium RNA in a foreign translation system.

To compare their sizes, ras proteins labelled in vivo were separated in polyacrylamide gels alongside ras proteins synthesized in vitro (figure 5). As expected, only the p23 ras protein was present in cells labelled in vivo during the first two hours of differentiation (lane 4). A lesser amount of p23 was present in cells labelled in vivo between 10
Figure 4. ras proteins immunoprecipitated from in vitro translations of developmental time-point RNAs from D.discoideum strain V12M2.

Total RNAs from the indicated time points were translated for 2 hours at 30C in a messenger-dependant rabbit reticulocyte lysate (MDL) containing 2 μC/μl of [35S]-methionine. 5 μl was removed from each 30 μl reaction volume, trichloroacetic acid (TCA) precipitated and counted. 75 μl of 259 Lysis Buffer was added to the remaining 25 μl of each lysate, which were then "precleared" with 50 μl of 10% RaRig Staph for 5 minutes as described in Methods. Each supernatant was then incubated with 5 μg of Oncogene Sciences Y13-259 or control antibody overnight on a rotator at 4C. 110 μl RaRig was added to each tube for 2 hours. Immunoprecipitates were pelleted and washed three times in 259 Lysis Buffer. After electrophoresis through a 14% polyacrylamide gel, the gel was dried and autoradiographed.
Figure 5. A comparison of the rates of migration of ras proteins synthesized in vitro and in vivo.

ras proteins were immunoprecipitated with the YL3-259 antibody from the following, and electrophoresed through a 16% polyacrylamide gel:

- Lanes 2 and 3: in vitro translated 2 hour RNA. Lane 2: no protease inhibitor added. Lane 3: 5 µg leupeptin added before and 5 µg added after translation.
- Lane 4: cells labelled in vivo from 0 to 2 hours. No protease inhibitors added.
- Lane 5: cells labelled in vivo from 10 to 12 hours. No inhibitors added.
- Lanes 6, 7, and 8: in vitro translated 12 hour RNA. Lane 6: 5 µg leupeptin added before and 10 µg after translation. Lane 7: 5 µg leupeptin added before translation. Lane 8: no protease inhibitor added.
- Lane 1: as Lane 2, but immunoprecipitated with YL3-238.
- Lane 9: as Lane 8, but immunoprecipitated with YL3-238.
and 12 hours of differentiation (lane 5). The p22 breakdown product was evident in the latter experiment, but p24 was not detected. This was consistent with the findings of Weeks and Pawson (1987). In contrast, both p23 and p24 were synthesized in vitro from the RNA of both 2 hour (lanes 2 and 3) and 12 hour cells (lanes 6-8). The p23 ras protein synthesized in vitro appeared slightly larger than the in vivo p23 ras protein. This slight shift is likely due to the fatty acylation of the ras protein only in vivo, which would reduce its apparent molecular weight in polyacrylamide gels (Buss et al., 1986). Ellis et al. (1981) found that the two murine Kirsten ras transcripts of 5.2 and 2.0 kb both produced the same size of p21 ras protein in vitro, and this protein also appeared slightly larger than the p21 protein seen in vivo.

The relationship between the two in vitro ras proteins was first examined by testing the ability of three protease inhibitors to alter the ratio of p23 to p24. Leupeptin and antipain were known to inhibit the degradation of p23 to p22 during the immunoprecipitation of ras proteins in Dictyostelium cell lysates (Weeks and Pawson, 1986). These protease inhibitors were added to immunoprecipitations of in vitro synthesized proteins in the event that Dictyostelium proteases synthesized in vitro had biological activity in rabbit reticulocyte lysates. When leupeptin (figure 5; lanes 3, 6 and 7) and antipain (figure 6, lane 1) were included prior to translation of the RNA, or PMSF prior to the overnight incubation of the proteins with Y13-259, (figure 6, lane 2) these protease inhibitors had no effect on the ratio between the two ras proteins. However, both antipain and PMSF reduced the amounts of two
Figure 6. Effect of protease inhibitors on ras proteins translated in vitro.

Lanes 1, 2, and 3: ras proteins immunoprecipitated from in vitro translation reactions of total 12 hour RNA. Lane 1: 5 µg leupeptin added at outset of translation, and 10 µg added at outset of immunoprecipitation. Lane 2: 10 µg phenylmethylsulphonylfluoride (PMSF) added at outset of immunoprecipitation. Lane 3: no protease inhibitors added.

Lane 4: ras proteins immunoprecipitated from AX-3 cells labelled in vivo from 0 to 2 hours of development. No protease inhibitors added. Proteins were separated by electrophoresis through a 16% polyacrylamide gel.
minor degradation products of 21 and 20 kd. The failure of all three protease inhibitors to effect the ratio of p23 to p24 suggests either that a degradation takes place which is not sensitive to these inhibitors, or that p23 is not derived by proteolysis from p24.

To further examine the relationship between the two ras proteins, RNA was translated in vitro for increasing lengths of time, to allow the detection of changes in the ratios of p23 to p24 in immunoprecipitates with increasing incubation time. An accumulation of the smaller protein over time would indicate that post-translational modification occurs in vitro, whereas a constant ratio would provide evidence that neither protein is derived from the other. This latter possibility seems to be the case. A constant ratio of the two proteins was observed between 10 and 120 minutes of translation of each RNA. Both proteins are translated in roughly equal amounts from vegetative RNA (figure 7), the larger species predominates in 2 hour RNA (figure 8), equal amounts are seen in 12 hour RNA (figure 9), and the smaller species is synthesized in larger quantities in 15 hour RNA (figure 10). These results suggest the two proteins are not derived from one another. If true, they must be translated from separate mRNAs. It cannot be determined from this work whether these two mRNAs are derived from the same gene (by utilization of alternate splice sites, or translational stop sites), or from two separate genes.

Amount of ras protein expressed throughout the developmental program:

A quantitation of the amount of p23/p24 synthesized in vitro was attempted, to better compare the amount of ras protein synthesized in
Figure 7. Ratio of ras proteins seen when vegetative RNA was translated in vitro for increasing lengths of time.

RNA was translated in a 300 μl volume, and at the times indicated aliquots were removed for immunoprecipitation with 5 μg Y13-259 antibody. A 125 μl aliquot, rather than the usual 25 μl, was removed at 10 minutes to compensate for the lesser amount of incorporation in this early time point (about 25% of the final incorporation at 120 minutes), and inadequate preclearing may have caused the high backgrounds seen.

Lane "c" (control): a 25 μl aliquot of the translation mixture was removed at 120 minutes and immunoprecipitated with Y13-238 antibody. Proteins were separated on a 16% polyacrylamide gel.
Figure 8. Ratio of ras proteins seen when 2 hour RNA was translated in vitro for increasing lengths of time.

2 hour RNA was translated in a 300 µl volume, and at the times indicated, 25 µl aliquots were removed for immunoprecipitation with 5 µg Y13-259 antibody, except for lane "c" (control) which was a 25 µl aliquot removed at 120 minutes and immunoprecipitated with Y13-258 antibody. No protease inhibitors were included. Proteins were separated on a 16% polyacrylamide gel.
Figure 9. Ratio of ras proteins seen when 12 hour RNA was translated in vitro for increasing lengths of time.

12 hour RNA was translated in a 300 μl volume, and at the times indicated, 25 μl aliquots were removed for immunoprecipitation with 5 μg Y13-259 antibody. "+" indicates the addition of 5 μg leupeptin immediately after translation. "-" lanes had no protease inhibitor added. Lane "c" (control) was a 25 μl aliquot removed at 120 minutes and immunoprecipitated with Y13-258 antibody. Proteins were separated on a 16% polyacrylamide gel.
Figure 10. Ratio of ras proteins seen when 15 hour RNA was translated in vitro for increasing lengths of time.

15 hour RNA was translated in a 300 μl volume, and at the times indicated, aliquots were removed for immunoprecipitation with 5 μg Y13-259 antibody. A larger aliquot, 125 μl rather than the usual 25 μl, was removed at 10 minutes to compensate for the lesser amount of incorporation in this early time point, as in figure 12. Lane "c" (control) was a 25 μl aliquot removed at 120 minutes and immunoprecipitated with Y13-258 antibody. Proteins were separated on a 16% polyacrylamide gel.
\textit{in vitro} to the levels of \textit{ras} RNA detected in Northern blots. \textit{ras} proteins were translated \textit{in vitro} from the total cellular RNAs used in the Northern blot shown in figure 1, immunoprecipitated from translation reactions with the monoclonal antibody Y13-259; then separated on SDS polyacrylamide gels (as shown in Figure 4) and quantitated. Quantitation of total \textit{ras} protein per translation reaction involved scintillation counting of p23/p24 bands that had been excised from polyacrylamide gels. The counts per minute found in each \textit{ras} doublet was divided by the counts per minute of [35S]-methionine incorporated into 5 ul of the \textit{in vitro} translation reaction of each RNA. A plot of the relative amount of \textit{ras} protein synthesized \textit{in vitro} from each RNA in two separate experiments (Figure 11) shows that the total amount of \textit{ras} protein synthesized in the rabbit reticulocyte system was greatest in protein translated from 2 hour RNA. The second greatest amount of \textit{ras} protein was translated from vegetative RNA, and lesser amounts of \textit{ras} protein were translated from the RNA of cells 5 to 15 hours into development (Figure 11). RNAs from early and late cells incorporated [35S]-methionine equally well in the \textit{in vitro} translation reactions, however the absolute amount of incorporation varied from one experiment to another. Some loss of total \textit{ras} protein resulted from stringent wash conditions and from "pre-clearing" with rabbit-anti-rat immunoglobulin-coated Staph. These steps were necessary to reduce immunoprecipitation backgrounds (data not shown). Nonetheless, the two experiments plotted in figure 11 were internally consistent with each other.

In summary, the pattern of expression of \textit{ras} appears different for
Figure 11. Plot of the relative amount of [35S]-methionine incorporated into p23/p24, relative to the total protein synthesized in vitro from the RNA of developing strain V12M2 cells.

Experiments were carried out as described in Methods. 5 μl of each 30 μl reaction were removed after 2 hours translation at 30°C, and the [35S]-methionine-labelled proteins were precipitated with trichloroacetic acid, washed and scintillation counted. The remaining 25 μl of translation mixture was immunoprecipitated with 5 μg Y13-259 antibody. Immunoprecipitates were washed, resuspended in 1x SDS sample buffer, and separated by electrophoresis through 12.5% polyacrylamide gels. The combined p23/p24 bands were excised and scintillation counted, and their percentage counts per minute (cpm) relative to the total cpm in immunoprecipitation volumes determined.
Northern blots of whole RNA (figure 1) and for ras proteins synthesized in vitro from whole RNA (figure 11). The predominant difference is that the greatest amount of ras protein is synthesized in vitro from the RNA of early developing cells, whereas the greatest amount of ras RNA is present in 12 and 15 hour cells. It is possible that vegetative and early ras mRNA is translated with very high efficiency, whereas later RNA, present in far greater quantities, is not. A second explanation for the abundant amount of ras protein in vegetative cells is that the Dd-ras cl probe and the Y13-259 antibody do not recognize the same gene products. Or perhaps the early abundant p24 is encoded by a second ras gene not detected by the Dd-rascl probe.

The pattern of proteins translated from the RNA of developing cells in vitro is also different from the pattern of ras protein immunoprecipitated from lysates of developing cells. The expression of the latter is essentially bimodal, with the larger peak of ras synthesis occurring in early cells, and the smaller peak occurring in 12 hour cells (Pawson et al., 1984). The reason for the absence of the second peak of ras when RNA is translated in vitro is unknown.

The two ras proteins in D.discoideum appear not to be related to one another by degradation or post-translational processing, suggesting they are encoded from two different mRNAs, which may be derived from either one gene or two.
DISCUSSION

It was of interest to compare the results of Northern blots done in this work to those of Reymond et al. (1984) who originally cloned the D.discoideum ras gene and published the first Northern analysis of its developmental expression. They analysed poly A+ RNA only, from strains AX-3 and NC-4. A 1.2 kb ras transcript was detected in variable amounts in AX-3 vegetative cells. Ras RNA was not detected again until 15 hours of development when both the 1.2 kb and a 0.9 kb species appeared, then increased to a maximum at 17 hours, and declined to zero by culmination. In the present work using strain V12M2, Northern blots of total RNA show that transcription of the 0.9/1.2 kb ras doublet increases steadily from 5 hours until 15 hours (Figure 1a) (with very low levels of ras RNA present at 2 hours and even less at 0 hours).

The lack of concurrence between the results of Reymond et al. (1984) and the present work may be due to strain differences. Reymond et al. (1984) found strains AX-3 and NC4 to differ in that NC4 lacked any early ras expression, so that the ras doublet first appeared at 12.5 hours. The strain used in the present work, V12M2 may differ from both AX-3 and NC4. A similar result to that of Reymond et al. (1984) for NC4 was found in the present work, except that low levels of the smaller ras species were detected in total NC4 RNA from 0 hours of development until the onset of pseudoplamodial stage, when much greater amounts of the ras doublet appeared. The detection of the early ras RNA in NC4 may be due to the greater sensitivity of detection obtained with the RNA probe (data
not shown).

The pattern of ras RNA expression differed from the pattern of expression of ras protein seen in vitro and in vivo. The greatest amount of protein was synthesized in vivo at 0 and 2 hours of development, when the least amount of ras RNA is detected, and a substantial but lesser peak of ras protein synthesis occurred in vivo at 15 hours (Pawson et al., 1985) when the greatest amount of ras RNA was detected in Northern blots in the present work. The greatest amount of ras protein was translated in vitro from the RNA of 2 hour cells, large amounts were translated from the RNA of vegetative cells, and relatively little from latter stages of development. It was not understood why the second peak of ras protein synthesis was absent in vitro, since radioactive label incorporation was comparable to that of RNA from earlier time points. There are several explanations for the lack of correlation between ras RNA levels detected using the Dd-rascl probe and the amount of ras protein immunoprecipitated from in vitro translations by the Y13-259 antibody. Firstly, the efficiency of translation of the ras mRNA might vary greatly throughout development, such that the small amount of RNA present in early developing cells is highly actively translated, whereas the abundant ras RNA present at pseudoplasmodial stage is translated very poorly. Secondly, the Dd-rascl probe and the Y13-259 antibody may recognize the products of different genes. Thirdly, perhaps the ras RNA detected by the Dd-rascl probe gives rise to a portion of the ras protein immunoprecipitated by the Y13-259 antibody, but a second gene not
detected by the Dd-rascl probe and actively translated in vegetative cells, and encodes the greater portion of the early stage ras protein, possibly the more abundant p24.

Interestingly, a 23 kd and 24 kd ras protein were synthesized both in vivo and in vitro, but their relative proportions and patterns of expression were radically different in these systems. The major species throughout development in vivo is the 23 kd protein, and the minor 24 kd species, when seen, is most abundant at pseudoplasmodial stage (Weeks and Pawson, 1986). In vitro, the major species translated from the RNA of cells from every time point except 15 hours was 24 kd (figure 4).

Tryptic peptide digests of D.discoideum ras proteins were carried out previously by Pawson et al. (1985). The pattern of [35S]-methionine-containing peptides separated by two dimensional electrophoresis were very similar for p23 in vitro, p24 in vivo, and p23 in vivo, although the first two species appeared to be the most closely related to one another.

Results of in vitro translation of Vl2M2 RNA in this work suggest that p23 and p24 are not related by post-translational processing or by degradation (figures 7 to 10). An apparent constant ratio of p23 to p24 was translated in vitro from each RNA tested, as determined by removing a sample of the proteins synthesized at intervals of a two hour in vitro translation. Alternatively, this data could result from a precursor/product relationship between p23 and p24, providing the rate of formation of the precursor was equal to the rate of decay of the product protein. This seems possible, since the half-life of p23 (reported to be 2.35 +/-
0.07 hours in early developing strain AX-2 cells) (Weeks and Pawson, 1987) would result in a significant loss of p23 over the 2 hour translation period. However, the protein processing would have to occur only during \textit{in vitro} translation, otherwise, levels of the product protein would accumulate during the overnight immunoprecipitation on ice. If the two ras proteins of strain V12M2 are derived from separate mRNAs, these mRNAs may be derived from two separate genes which are expressed differentially throughout development, or the two RNAs may be derived from the same gene by alternative splicing or perhaps by alternate transcriptional start sites, such as seen in the \textit{Drosophila} alcohol dehydrogenase gene (Benyajati et al., 1983). The latter possibility requires that these alternate sites be utilized in different proportions \textit{in vitro} than \textit{in vivo}, since the relative amounts of p23 and p24 differ \textit{in vitro} and \textit{in vivo}.

The relationship between the two ras proteins and the two ras RNA species detected is presently unknown. It seems possible that p24 may be derived from the the 1.2 kb ras transcript, and p23 from the 0.9 kb transcript. However, this model cannot apply to the early stages of development, when very low (or zero) levels of just one ras RNA species is detected, yet large amounts of p24 and p23 are translated from the same RNA stocks \textit{in vitro}. This suggests either that a second ras transcript exists undetected in early stage RNA, or as previously suggested, that the RNA probe and the Y13-259 antibody do not recognize the same gene products.
The human Kirsten ras gene gives rise to two mRNAs by alternate splicing of the 3' exons "4A" and "4B", and the proteins encoded by these RNAs are slightly different in size (McGrath et al., 1983). By analogy, both of the Dictyostelium ras proteins may be derived from the one known ras gene if it contains alternate splice sites within the known exons, or if a hitherto undiscovered exon exists outside of the sequenced region of the ras gene. This hypothesis also requires that the ratio of alternate splice sites utilized changes during development. The unusually high levels of p24 in vitro may be aberration caused by translation of Dictyostelium RNA in a rabbit reticulocyte lysate.

In addition to the expected D.discoideum ras restriction fragments, additional ras-homologous bands were detected in genomic AX-3 DNA under conditions of low stringency (30% formamide, 42C). The bands may represent more than one heterologous ras genomic sequence. Transcripts derived from these heterologous ras sequences should be detectable in Northern blots under similar hybridization conditions. No new ras-homologous RNAs were detected in low stringency Northern blots of vegetative RNA to account for the large amount of protein synthesized at this stage. Hence, there is at present no good evidence to suggest that the new ras-homologous genomic sequence is transcriptionally functional.

An interesting correlation was noted between the peaks of ras protein expression, and the peaks of mitotic activity that occur during the developmental program. Zada-Hames et al.(1978) found that two peaks of mitotic activity occurred during the D.discoideum developmental program,
centered over 4 and 16 hours. Furthermore, [3H]-thymidine uptake assays indicated that two periods of DNA synthesis also occurred during development, and coincided with the two periods of mitosis. It was estimated from autoradiographs of disaggregated fixed cells, that only 25% of cells incorporated 3H-thymidine in the first period of mitosis/DNA synthesis, during which 47% of the cell population underwent mitosis; and only 10% of cells incorporated 3H-thymidine in the second period, at 16 hours, when 25% of the cell population was undergoing mitosis. Pawson et al. (1985) found two peaks of ras protein expression in vivo, centered over 1 and 12 hours, with the peak at 1 hour always greater than the peak at 12 hours. Thus during development, a peak of ras protein expression precedes by about 3 hours, each peak of mitosis and DNA synthesis. This is interesting in light of the mounting body of evidence that suggests ras is involved in signaling cells to enter S phase (Stacey et al., 1984; Feramisco et al., 1984; Mulcahy et al., 1985).

Further work to establish if the two ras proteins are linked to mitosis, or differentially linked to cell type, will lead to an increased understanding of the role ras plays in the cell cycle.


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