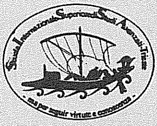

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Positive and Negative Pathways in Cell Proliferation Control

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-

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INTRODUCTION

1. The basal eukaryotic cell cycle: yeasts.

The cell cycle represents the complete process of DNA replication, mitosis and cytokinesis that leads to the production of two daughter cells from a single mother cell. This cycle is tipically divided into four phases. The events of DNA replication (S phase) and mitosis (M phase) are separated by gaps of variable lengths named G1 and G2. All cell types undergo some versions of this basic cycle, although differences in the replication and in the timing of the gap phases exist (11).

The budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe* are simple single celled fungal Eukaryotes with a DNA content only four to five times greater than that of *E. coli*. Despite their simplicity, they bear most of the basic features typical of more complex Eukaryotes (56). The two yeasts are as evolutionary divergent from one another as either from mammalian cells. *S. cerevisiae* modulates its cycle mainly at G1/S, while *S. pombe* regulates its cell cycle progression at G2/M. Genetic studies have however revealed that each yeast contains both control points even though one is usually cryptic in rapidly growing cells.

S. cerevisiae cells grow as a stable haploid or diploid cell and produce a small bud which, after initiation, grows continuosly through the cycle. The nucleus migrates into the bud neck and there undergoes mitosis although the nuclear envelope

remains intact, and there is no apparent chromosome condensation. Thus the duplication of the spindle pole bodies and the reorganization of interphase cytoplasmic microtubules into a mitotic nuclear spindle must take place very early to allow bud formation and migration of the nucleus to the bud neck. For this reason during most of the cell cycle cytological markers of late G2/M are visible. Consequently there is no clear definition between S, G2 and M phases and mainly the budding yeast regulates its cycle in G1. The regulatory network coordinates the division machinery by monitoring environmental constraints such as concentration of nutrients and cell size during G1 phase. Infact during G1, the yeast cell has three choices: 1. it can proceed through the cell cycle and divide; 2. if nutritionally deprived, it can enter into a stationary phase; 3. if the cells are haploid they can mate. During conjugation, two cells of opposite mating type (a and α) synchronyze their cell cycles with a transient arrest at G1 in response to peptide mating pheromones. Two haploids will then be able to fuse to form a stable dyploid, which will continue to divide. If a dyploid cell is starved of nutrients, however, it has the option of undergoing a meiotic cycle to produce four haploid spores.

The fission yeast *S. pombe* has a cell cycle more similar to that of higher cells having distinct G1 and G2 phases. However in rapidly growing cells, S phase begins before cytokinesis is complete, and there is only a very short G1 phase. This reflects the fact that under these conditions *S. pombe* exerts cell cycle control mainly at the G2/M transition where information about cell size and nutrient concentration is transduced. If cell size is too small, the fission yeast extends its G1 phase to allow the

cells to reach a critical size before entry into S phase. *S. pombe* cells continue to grow until nutrients become depleted. When this happens, cells have two choices: they may arrest either in G1 or G2. If cells of both mating types are present, however, the fission yeast will undergo conjugation to form a diploid cell which will sporulate immediately, since diploid form of *S. pombe* is very unstable.

Many strains have been isolated carrying ts mutations that arrest the cell at a specific point in the cell cycle. These cell division cycle (cdc) mutants have allowed the identification of a variety of functions necessary for cell cycle progression.

1.1 START.

Hartwell and colleagues defined the concept of START in budding yeast. START occurs during G1 regulating the G1/S transition and is usually considered the point of commitment to the cell cycle. After START, the cell must initiate DNA replication and complete a round of division. Passage through START determines the rate of progression through the cell cycle and before passing it, cells must have a critical cell mass. Thus START is a major rate limiting step during the cell cycle. In S. cerevisiae the master control gene involved in START is CDC28, which, if mutated, blocks the cells at the G1/S transition. Because some alleles of CDC28 are also able to block the G2/M transition, this gene is involved in the regulation of both points in the cell cycle. CDC28 is a functional and sequence homologue of the *S. pombe* $cdc2^+$ gene that, once mutated, also blocks both the G1/S and G2/M transitions. The $CDC28/cdc2^+$ gene encodes a 34

Kd serine/threonine protein kinase ($p34^{cdc2}$). Homologues of the $p34^{cdc2}$ kinase have been identified in all the Eukaryotes tested. Moreover many of these kinases have been shown to be functional homologues of the yeast protein, since they are able to rescue the mutated phenotypes. This functional conservation strongly suggests that at least parts of the controlling networks at the G1/S and G2/M transitions must be conserved in higher Eukaryotes.

Other genes at the G1/S transition have been identified by screening for mutations that allow conjugation when cells are arrested. In *S. pombe*, mutants in the $cdc10^+$ gene arrest cells before START. This gene is homologous to many different proteins such as the transcriptional regulators of mating type switch in budding yeast SWI4 and SWI6 and developmental genes in *Drosophila melanogaster* and *Chaenorabditis elegans*.

Other genes acting in late G1 and during the G1/S transition in budding yeast have been recently cloned and characterized. If these genes are mutated, cells arrest before the initiation of DNA replication but are unable to undergo conjugation thus being blocked after START. Suppressor analysis has identified several genes: one of them, csk1, is highly homologous to $suc1^+$ protein of the fission yeast also showing a similar suppressor effect in $cdc2^{ts}$ mutants that act at the G2/M boundary.

Other two interesting suppressors are represented by CLN1 and CLN2 genes. They have sequence homology to a family of proteins called cyclins, first identified in marine invertebrates. Sequence comparison identified a third G1 cyclin, named CLN3 and originally identified as WHI1 because of its ability to accelerate the cells through G1 at a reduced size. Deletion of one of them, or

two of them in the same cell, is not lethal. The three G1 cyclins are functionally redundant and are able to alter the rate of progression through START. A fission yeast gene able to substitute for budding yeast CLN3 mutation has also been cloned (puc1+). Thus a G1 specific protein complex, between CDC28 and the G1 cyclin analogous to that described in G2/M transition (see below) does exist in yeast cells. This S-phase Promoting Factor (SPF) must provide an interface with important controls required for G1 progression. It seems that a complicated array of signals from the mating pheromone response pathway and the nutrient sensing mechanisms is integrated through the G1 cyclins, so that while anyone of the three responds to a subset of signals, the system is able to respond appropriately to any change in the environment. G1 cyclins have only 25% homology to the mitotic cyclins but at least one G1 cyclin, CLN2, has been shown to bind and activate $p34^{CDC28}$. The activity of the $p34^{CDC28}$ at START appears to be regulated only by the level of the activating cyclin subunit and not by phosphorylation as for $p34^{cdc2}$ at the G2/M transition. Two of the G1 cyclins, CLN1 and CLN2, have very short half-lives, their level being determined by the rate of transcription. On the contrary, CLN3 is present at very low and constant levels throughout the cell cycle (37).

The expression of at least 17 genes encoding the enzymatic machinery for DNA synthesis is restricted to the late G1 and S phase of the cell cycle; these genes include DNA pol. I, DNA pol. III, the yeast homologue of PCNA, the large and small subunits of DNA primase and DNA ligase (7). This coordinate induction suggests that their expression may be governed at the transcriptional level by a common factor. The upstream regions of

each of these genes presents at least one copy of the sequence 5'-ACGCGTNA-3' which contains the recognition sequence for the MluI restriction endonuclease. This sequence has been shown to be sufficient for conferrring cell cycle regulation to a constitutive promoter: it has been named MCB (MluI cell cycle box) (135).

The HO gene, responsible for the mating type interconversion, is expressed only in haploid mother cells and before DNA replication. The peak of HO transcription coincides with that of the DNA synthesis genes. This pattern is determined by another element (5'-PuNNPy-CACGAAAA-3') named CCB (cell cycle box) because it acts as a positive regulatory sequence and is sufficient to confer cell cycle replication. CCB is also present in CLN1 and CLN2 promoters.

SWI4 and SWI6 gene products are components of a factor (SBG) that bind the CCB sequence element responsible for the activation of the HO and of the CLN1 and CLN2 genes during late G1. Moreover a replication factor (MBF) containing SWI6 and a 120 Kd protein is able to bind the MCB sequence responsible for late G1 transcription of DNA replication genes (8) (24) (138) (167).

Since active $p34^{CDC28}$ increases CLN transcription and as CLN synthesis increases, more $p34^{CDC28}$ would be rescued to the activated form, two groups have recently proposed that they constitute a positive feedback loop in CLN1 and CLN2 transcription (38). Thus once activated, this transcription loop ensures that START is irreversible and the loop is turn off only after entry into S phase when CLN1 and CLN2 transcriptions are inhibited and the proteins degraded. It was thus suggested that $p34^{CDC28}$ might enhance CLN transcription by phosphorylating SWI4 and/or SWI6 to increase their activity.

In the fission yeast, only one of the DNA synthesis genes, cdc22+, is periodically expressed in late G1. The promoter region of cdc22+ has two MCBs thus suggesting that a similar control on DNA synthesis genes could occur in fission yeast. Recently it has been shown that a complex, containing the cdc10+ gene product is able to bind this factor. This result is particularly interesting since cdc10+ gene is required for START in fission yeast and is highly homologous to SWI4 and SWI6. Thus an involvement of MCB DNA binding domain in transcriptional control at START may be a conserved feature of eukaryotic cells.

The mating factors of yeast are extracellular proteins that induce a variety of responses such as arrest at START, morphological changes and transcriptional induction of several genes. The signalling pathway by which cells respond to mating pheromones present 4 main steps: a receptor (STE2 or STE3, depending on whether the cell is an a or an α cell), a G protein (with GPA1, STE4 and STE18 subunits), a group of protein kinases (STE7, STE11 and FUS3) and a transcriptional activator (STE12). The final result of the activation of this transcription factor is the induction of many genes, one of them is FAR1. This gene has been shown to be absolutely required for cell cycle arrest as elicited by pheromone treatment. This property is probably the effect of CLN2 activity inhibition. This control, elicited on cyclins, is another example of how different signals can be transduced at this point of the cell cycle (27) (114).

1.2 G2/M transition.

Several genes acting near the G2/M transition have been identified in *S. pombe*. The $p34^{cdc2}$ protein kinase is essential for the onset of mitosis both in *S. cerevisiae* and *S. pombe*. Other genes have been cloned by suppressor analysis that identify interacting functions. Three temperature-sensitive alleles of the $cdc13^{+}$ gene were isolated as suppressors of a $cdc2^{ts}$ mutant. The $cdc13^{+}$ gene is essential: when deleted, cells block at G2/M. $cdc13^{+}$ has homology to the B-type class of mitotic cyclins. In *S. cerevisiae* four B type cyclins have been isolated: CLB2 appears to be the most important, CLB1 and CLB3 both have significant effects while CLB4 have very little effects. The $Suc1^{+}$ gene was also isolated as a high-copy suppressor of $cdc2^{ts}$ and appears to be necessary for the successful completion of mitosis.

A mutated $cdc25^{+}$ gene blocks cells in G2 while overexpression of this gene advances cells into mitosis: $cdc25^{+}$ thus acts as a dosage dependent inducer of mitosis. A functional homologue of $cdc25^{+}$ in *S. cerevisiae*, MIH1, has been isolated on the basis of its ability to complement the *S. pombe* mutation. Moreover genetic analysis designed specifically to search for rate-limiting genes in *S. pombe* has identified mutations in which cells progressed through cell cycle at a reduced size as expected when the timing of entry into mitosis is accelerated. One of these mutants identified the $wee1^{+}$ gene. While a mutation removing $wee1^{+}$ gene function leads to cell division at an abnormally small size (wee in Scottish means small), overproduction of wt $wee1^{+}$ product produces an elongated cell so that $wee1^{+}$ acts as a dosage-dependent inhibitor of mitosis. The effects of $wee1^{+}$ and

cdc25+ are additive, suggesting that they work independently. Thus there appears to be a biforcation in the upstream control of cdc2+ with two converging independent pathways. An additive mutation in the wee1+ repressive part of this pathway has been defined as a negative regulator of this gene and it has been named nim1+.

The overall amounts of cdc2+ remain constant both at the mRNA and protein level in both yeasts but its activity is dramatically increased at the onset of mitosis and determines the timing of entry into M. This activity is regulated by phosphorylation and multiprotein complex formation. The protein is infact partially dephosphorylated on Threonine and Tyrosine upon entry into and during progression through mitosis: the G2/M transition has been shown to be dependent upon Tyrosine dephosphorylation. Moreover there is a direct association between cdc13+ and cdc2+ gene products. The cdc2+/cdc13+ complex in yeast is homologous to the MPF (Maturation Promoting Factor) activity first identified in Xenopus. Cyclin is required for entry into mitosis and levels of cdc13+ protein abruptly decrease as cells pass through mitosis. After passing START, cells begin to synthesize cyclin B which accumulates and binds to $p34^{cdc2}$. This complex is inactive since binding of cyclin B to $p34^{cdc2}$ induces phosphorylation of Tyrosine 15 of $p34^{cdc2}$ which inhibits its protein kinase activity. The inactive complex is dephosphorylated on Threonine 160. This phosphorylation is required for MPF activity but is not sufficient to overcome the inhibitory effect of Tyrosine phosphorylation. The subsequent removal of the Tyrosine phosphate from $p34^{cdc2}$ during late G2 activates MPF and leads to induction of mitosis. These postranslational reactions thus play a

major role in the timing of mitosis. The Tyrosine kinase was identified as the product of the fission yeast wee1+ gene whereas the phosphatase is the product of cdc25+ gene. When formed, MPF is able to induce chromosome condensation, nuclear envelope breakdown and assembly of the mitotic spindle both directly and by activating other effectors.

1.3 Checkpoints.

Feedback controls must have at least three components: a sensor that monitors the completion of the downstrem event, a signal that this sensor generates and a response element in the cell cycle engine. START and entry into mitosis are the major checkpoints where feedback controls regulate the cell-cycle machinery sensing both cell size and the availability of nutrients and growth factors. Weinert and Hartwell pioneered the genetic analysis of feedback controls by analyzing radiation-sensitive mutations in budding yeast. They showed that the defect in rad9, rad17 and rad24 resides in their ability to arrest the cell cycle in response to damaged DNA. Deletion of the RAD9 gene increases the rate of chromosome loss 20 fold. Two other mutants mec1 and mec2, are also defective in their ability to arrest cell cycle both in the presence of damaged or unreplicated DNA thus suggesting that although different sensors detect the same event, both feedback controls converge and act in the same checkpoint. The response to DNA damage has also been studied in the fission yeast where a number of rad genes allows cells with damaged or unreplicated DNA to enter mitosis. A fission yeast mutation in the pim1 gene also affects this process. Mutant cells can enter

mitosis even when they have not passed START and this phenotype can be suppressed by overexpression of a small G protein. In general if S phase is blocked by inhibitors of DNA synthesis, M phase does not occur. Overexpression of cdc25 is correlated with increased Tyrosine phosphorylation of cdc2 and results not only in premature entry into M phase but also in the relief of M phase dependence on completion of S phase.

1.4 The relationship with cell proliferation control: quiescence in yeast.

Close comparisons are often made between the stationary phase state of yeast and the G0 state of metazoan cells; indeed the non-growing cell is sometimes referred to as being in G0 phase. However this analogy should be treated with some caution. In mammalian cells, growth control is subject to control of a cellular society within a multicellular system. In general this situation does not apply to a free-living multicellular organisms such as yeast. A mammalian cell in G0 is deprived of growth factors rather than of basic nutrients and is stable in an active metabolic state. In contrast there is no evidence that growth factors have any role in growth control in yeast: the yeast cells in stationary phase are starved of basic nutrients and are metabolically dormant.

2. Cell cycle in higher Eukaryotes: a large increase in complexity.

2.1 G2/M transition.

Considerable evidence now exists that control of mitosis is fundamentally similar in all Eukaryotes. Entry into mitosis is determined by the activation of an M phase specific protein kinase, originally known as M phase Promoting Factor, the highly conserved activity capable of inducing M phase when introduced into Xenopus oocytes or in activated egg cell-free systems. The M phase specific kinase is composed of two subunits. The catalytic subunit is the highly conserved 34 kd protein kinase, $p34^{cdc2}$ and the regulatory subunit is cyclin B, that is one of the related proteins called cyclins, originally discovered as proteins that strongly accumulate during interphase but become destroyed at each mitosis during early invertebrate embryonic cell divisions. Moreover mitotic cyclin mRNAs will induce M phase in Xenopus oocyte on activated egg extracts. Cyclin destruction at the end of metaphase is absolutely required for exit from mitosis. There are at least two types of mitotic cyclins, as defined by sequence homology, in the central 150 amino acids of the protein, an A and B type. In some species, including humans and Xenopus, there are two closely related B-types B1 and B2.

2.2 START.

The most obvious requirement for the onset of cellular S phase is the completion of the preceding mitosis (39) (25). Prior

starting DNA synthesis, during the G1 phase, the chromosomes become competent for DNA replication; infact fusion of a G1 cell to an S phase cell prematurely induces DNA synthesis in the G1 nucleus. In contrast, parallel experiments show that a G2 nucleus will not rereplicate its DNA after fusion of a G2 cell to an S phase cell. This is not due to the presence of a diffusible inhibitor in G2 cells, since replication in the S phase nucleus is unaffected. Rather, the block to rereplication must be a cis-acting signal that specifically marks the G2 chromosomes or G2 nucleus (157). Thus the relationship between M phase and S phase is tightly dependent on resetting the mechanism that limits the replication of cellular DNA to exactly once in every cell cycle. Two different models have been proposed to explain these cellular properties: one model postulates that all factors necessary to replicate DNA can remain continuously in excess through S phase, but that a cis- acting signal, marking replicated DNA prevents its rereplication. A second model postulates that the limiting abundance of an essential replication factor assures that DNA sequences will be replicated only once per S phase. The second model has originated from studies on replication of nuclei in cell free extracts derived from activated Xenopus eggs. In the presence of cycloheximide nuclei incubated with Xenopus egg extracts would replicate all their DNA exactly once; in the absence of protein synthesis inhibitors periodic nuclear envelope breakdown and reformation occurs and this process is followed by a complete round of semi-conservative synthesis occuring in each nuclear cycle. Thus an event occuring at mitosis was sufficient to render the genome capable of a new round of DNA replication. This event was mimicked by unphysiological, chemical or enzymatic

permeabilization of the nuclear membrane and thus suggested that the nuclear membrane might act as a barrier to a limiting replication initiation factor (licensing factor). Thus if each licensing factor could be used only once to start DNA replication, this would prevent reinitiation of DNA synthesis until a new factor is in contact with DNA after mitosis. However this model, as another similar model that evokes depletion of essential replication factors during S phase, is able to explain regulated replication just in a situation where initiation occurs synchronously throughout the entire genome. On the contrary in somatic cells it appears that initiation events occur at different times through S phase. This implies that all necessary replication factors remain active through most or all this period. A dramatic demonstration that the availability of replication factors cannot fully explain the control of DNA replication in somatic cells was provided fusing G1 cells to metaphase cells in the presence of colcemid. The mitotic cell was able to induce G1 nuclear membrane breakdown and premature chromosome condensation of the G1 DNA, so that the two genomes shared a common cytoplasmic environment. Remarkably, cells entered a kind of S phase in which the G1 chromosome began to duplicate their DNA in their condensed form and in the absence of an intact nuclear structure. Since the metaphase chromosomes in the same cytoplasm did not replicate, these experiments prove that a mechanism preventing rereplication of DNA can still survive despite nuclear membrane breakdown. In similar experiments, it was found that DNA injected into CSF arrested Xenopus egg would replicate exactly once while the endogenous chromosomal DNA does not replicate. These results imply that a cis-acting negative

replication control system functions by tagging DNA that have already replicated. This tag may reside on the DNA itself or can partecipate in a replication dependent change of the chromatin structure.

However in most cells completion of mitosis and the erasure of rereplication controls are not sufficient for the initiation of the S phase. Infact many eukaryotic cells don't replicate their DNA despite of having a long G1 phase where the nucleus is competent to replication since it will rapidly initiate DNA synthesis if fused to an S phase cell. Thus one of the major conclusions, drawn from mammalian cell fusion studies, is that a limiting trans-acting inducer of S phase gradually accumulates during G1. Infact in many different experimental conditions all nuclei within a single heterodykarion entered S phase together. The properties of the S phase inducer bear some similarities to cyclins, and may suggest that a cyclin-like activity is important in regulating the onset of DNA replication.

By probing extracts from cells at defined positions in the cell cycle, it was shown that S phase extracts were at least 20 fold more efficient in initiating SV40 viral DNA replication than G1 extracts. Moreover extracts prepared from G2 cells were active, while extracts from mitotic cells were inactive. These observations suggest that all factors necessary to replicate DNA might remain active through S phase and during G2. The multiprotein complex responsible for this effect was purified and was shown to contain the human homologue of cdc2 protein: this protein was also present in G1 but the extract was inactive. Infact addition of recombinant cyclin A was able to activate a cdc2/like activity and replication. These results suggest the

existence of an SPF/like structure present in higher eukaryotic cells (20) (41). However, even if these data suggest a role for the p34 kinase in activating DNA synthesis, they do not exclude participation of the cdc2/like complex in earlier events that result in commitment to S phase. Multiple intra- and extra-cellular signals may thus be transduced at the level of activation of different cdc2/like proteins at various points during G1 progression.

The increase in complexity in the metazoan cells is clearly visible when considering the large number of possible cyclins and cdc2/like proteins that can form different multimolecular complexes.

Putative G1 cyclins have been isolated because of their ability to complement the knockout of CLN1-3 in S. cerevisiae: cyclin C, cyclin D and cyclin E (95) (180). They show significant homology to both the CLNs and the mitotic cyclins. Cyclin C mRNA increases transiently in early G1 phase, the amount of cyclin E mRNA peaks sharply just before S phase and cyclin D has been previously described as a mitogen responsive gene (CYL1). Moreover PRAD1, isolated because overexpressed in parathyroid tumors, and bcl1, translocated and overexpressed in human lymphomas, are also D-type cyclins.

Cyclin A and E are able to overcome pRB-mediated suppression of proliferation by activating its phosphorylation. Moreover cyclin E and cyclin A has been found to be complexed with E2F-p107 complex in a cell cycle specific manner (86) (112) (166).

Recently the role of cyclin A in the establishment of S phase has been finely demonstrated. Microinjection of antisense cyclin

A mRNA or affinity purified anti-cyclin A Ab during G1 phase inhibits the entry in S phase while interaction with cyclin B has no effects. Its role in proliferation control is also suggested by its association with adenovirus E1A gene product in adenovirus-infected cells. Moreover the hepatitis B virus was found to be integrated in the cyclin A gene in a hepatocellular carcinoma (119).

During the past years more than 30 genes that are related to cdc2 have been identified and cloned. One subgroup, consisting of the most related proteins, shows up to 70% aminoacids sequence identity with cdc2. One subset, however, consists of polypeptides that contain only the ~300 aminoacids residues of the conserved protein kinase domain. Within this subset, all cdc2s from different species as well as cdk2, cdk3, PSSALRE and PSK-JB are included. The cdc2s and cdk2s have been shown to be associated with different cyclins that act as regulatory subunits and probably other members with this subunit will associate with cyclins.

Various combinations of cyclins and cdk2s thus provide the diversity of possible kinase activities offering both unique levels of control and regulatory points in the timing of activation, substrate specificity and susceptibility to various activating and inhibiting events.

3. Cell proliferation control: a higher hierarchy.

In multicellular organisms the proliferation state of a cell is the result of a very complex network of regulatory signals (145) (121). Hormones, growth and cytostatic factors are produced and released from the different districts of the body or from adiacent cells. They allow a correct development and differentiation of the right cell type, at the right space, at the right time. Different extracellular matrix components are also able to interact in different ways with cellular receptors and induce activating or inhibiting growth transducing pathways. However the complex formations between ligands and membrane or nuclear receptors are only the first step of a cascade of events that allows large changes in gene expression and phenotype. The correct execution of the genetic program is continuously checked during all phases of the cell cycle by integrating growth inducing and inhibiting pathways, positive and negative regulatory elements of cell cycle machinery and cell-type specific gene expression.

Using various *in vitro* systems, a general model for cell proliferation control has been designed. According to this, four major states have been defined: growing, quiescence, apoptosis and differentiation. During the last years the molecular features of these different states have started to be dissected thus revealing the relationships and the mechanisms of the switches that differentiate these states.

In growing cells, the proliferation control pathway monitors that all the intra- and extra- cellular conditions are able to support growth. A key regulatory molecule involved in this

process is the c-myc gene product. The presence or absence of this protein is tightly connected with the growth state of the cell: its level is high in proliferating cells and undetectable in growth arrested cells.

Infact if proliferation restrictions are present when a cell is in an actively growing state, c-myc is immediately down-regulated. However these cells continue to grow until the next postmitotic phase where both internal and external conditions are checked for restrictions and effectively transduced into the cell division program. This state is reported as G1u (G1 uncommitted). If all the conditions necessary for growth and division are present and successfully verified (c-myc present), the cell can commit itself to a new cycle, termed G1c (G1 committed), that is the cell can pass through START. Conversely, if in G1u the conditions mentioned above are not satisfied (c-myc absent), the cell has the option to exit from the cell cycle and enter into the quiescence or a predifferentiative postmitotic state (171) (181).

Quiescence (G0) appears to be the only reversible 'not growing' state. Infact if growth factors are added to quiescent cells, they are able to leave G0 and enter into the S Phase. The mitogenic pathways elicited by growth factor involve the signal transduction cascade from membrane to nucleus that results in the induction of a gene expression pattern largely specific for the G0->G1 transition. Later on other genes are activated and this pathway finally crosses with the G1 cell cycle machinery. At the same time the growth arrest specific genes must be downregulated and the activity of their products must be neutralized. Infact the cell does not normally enter S phase if the quiescent pattern of gene expression is not erased. This could be

the result of a checkpoint control that is elicited by other classes of growth regulatory genes among which the best characterized is p53. Other growth inhibitory elements are probably involved at the very beginning of the transition from the growing to the arrested state. Nuclear proteins like Rb are candidate genes to sustain this regulatory role. It has been recently shown that growth activating pathways involving c-myc and growth inhibiting pathway involving Rb as key molecules are functionally interfacing.

Both *in vitro* and *in vivo*, the relationship between the growing state and differentiation has recently become more clear: genes that are able to induce growth must be repressed to allow exit from the cell cycle and genes that code for differentiation factors are able to inhibit cell proliferation.

Moreover when conditions are not favorable for survival or when social restrictions in multicellular organisms invoke, cells enter a programmed cell death-suicide pathway. This final pathway seems to be intrinsically related to proliferation control since c-myc is able to induce apoptosis when expressed under conditions that can not support growth.

3.1 The growing state: a central role for c-myc.

In non-trasformed cell cultures, c-myc expression is strongly responsive to mitogens and other environmental signals (102) (126) (131) (133). Infact when considering the transition from growth arrest to a fully proliferating state, c-myc mRNA/protein expression is regulated at two different levels. During G0/G1 transition c-myc mRNA/protein is induced at least

40 fold in different cell lines/types (lymphocyte, BALB/c3T3, NIH3T3) in response to different stimuli (antigen binding, serum or single growth factor) (9) (35). Later on this amount drops to a still significant level during all the stages of the cell cycle in a growing cell culture. This two step response probably reflects two different involvements of c-myc in signal transduction and proliferation control. c-myc gene belongs to the class of 'immediate early' response genes that are induced by mitogens in the absence of protein synthesis.

Although induction of c-myc protein in confluent fibroblasts occurs with essentially the same pattern as in sub-confluent cells after fresh serum addition, expression is not maintained and falls to undetectable levels after about 12 hours in confluent cells where it appears to be down-regulated in late G1-early S Phase by the dominant antiproliferative effect of contact inhibition (171).

Most if not all the 'immediate early' response genes are selectively expressed during G0/G1 transition, on the contrary c-myc is continuously present, albeit at a lower level, in actively proliferating cells throughout all the cell cycle phases. Moreover in fibroblasts the 'proliferating level' of c-myc depends on the presence of mitogenic factors: removal of growth factors, at any point in the cell cycle, causes a rapid disappearance of c-myc protein except during mitosis. It has been suggested that the rapid disappearance of c-myc mRNA/protein in response to growth factor deprivation or density dependent inhibition constitutes a major signalling mechanisms. Infact c-myc seems to represent the integration point of a range of positive and negative growth-regulatory signals to which cells must respond.

Thus a fall in c-myc protein levels at any time during cell cycle causes arrest at the next available point in the cycle; if c-myc protein levels fall while the cells are already in postmitotic G1, they arrest immediately, on the contrary they proceed until they enter the G1 of the next cell cycle and arrest there. Repression of c-myc expression in the absence of growth factors is a prerequisite to suppress inappropriate cell proliferation.

3.1.1 c-myc gene structure and function.

The myc oncogene was first identified in the myelocitomatosis virus 29 (MC29), a replication defective avian retrovirus. This virus induces acute transformation of a wide variety of cells in vivo and in vitro, including fibroblasts, epithelial and myeloid cells.

Three additional replication-defective, oncogenic avian retroviruses (CMII, OK10 and MH2) have been classified in the same class as MC29 since they also posses common myc sequences.

The v-myc gene for MC29 was isolated as a gag-myc fusion product consisting of 1358 bp of gag sequences linked to 1568 base pairs of the myc gene.

The structure of c-myc gene is very conserved among Vertebrates but it is not present in lower Eukaryotes.

It consists of three exons. The first exon contains the untranslated region and is unusually long (400 bp). The two next exons comprise both the open reading frame and the 3' untranslated region.

During the last years a lot of work has been performed to understand how the regulation of myc proto-oncogene occurs, the sequence involved in these different mechanisms and what key regulatory steps are targets for the deregulated expression of myc in tumoral cells.

c-myc expression has been reported to be modulated at the transcriptional, postranscriptional and translational level depending on the cell type and the nature of the stimulus.

Control of c-myc transcription occurs at the level of both initiation and elongation. The c-myc gene is transcribed from two distinct promoters, P1 and P2, located close to each other (162 nucleotides apart in the mouse gene) (130). The relative levels of P1 compared to P2 initiated transcripts are sometimes modulated even if the reason for the existence of the two promoters are not known. In addition, transcription from an upstream promoter, termed P0, has been observed in some cell types (129).

The responsiveness of c-myc to serum and single growth factor occurs through the P2 promoter. Together with a TATA box, the region between P2 and P1 contains three binding sites for different cellular proteins: ME1a2, E2F and ME1a1 (from 5' to 3').

While the ME1a2 site binding protein has been recently cloned (JP1) and it resulted to contain a zinc-finger domain, the E2F binding site has resulted to have a central role in cell proliferation and cell cycle control and the recent cloning of the gene coding for the protein that binds to this sequence will help its characterization.

Measurement of the rate of transcription across different regions of the myc gene has suggested that the expression of the myc mRNA may be determined by modulation of the transriptional

elongation through a block near the 3' end of the first exon of the myc gene. The mechanism by which transcriptional termination or pausing is used to modulate the expression of known sequences is commonly named 'attenuation'. Moreover when a 180 nt fragment derived from the 3' end of exon 1 is inserted into an intron of the human α1-globin gene, it is able to partially block transcriptional elongation by RNA Polymerase II. The increased level of c-myc in Small-Cell Lung Cancer cells has resulted to be a consequence of the loss of transcriptional attenuation (179).

Postranscriptional regulation involves mRNA stability. The normal c-myc mRNA half-life is very short from 10' to 30' depending on the cell type (12).

In vitro induction of c-myc in the presence of a translational inhibitor like CHX results in the stabilization of its transcripts. This phenomenon has been explained by the inhibition of the translation-dependent poly(A)+ tail shortening (91) (162). The first step is followed by a translation independent step that induces rapid degradation of the body of the mRNA possibly proceded by the removal of the short remaining of the poly(A)+ tail. Accordingly it has been shown that the rate of c-myc mRNA degradation is probably different in growing and differentiated cells. Infact in growing cells a large part of c-myc transcript is stored in a non-adenylated form that is intrinsically more stable. It is not clear what sequences are involved in this process. The role of the AUUUA rich species present in the 3' UTR region, known to confer mRNA instability, is still controversial (21): in some systems mutations of this region does not have any effect, while a 3' truncated form of c-myc, caused by chromosomal translocation in a human T-cell leukemia, increases mRNA

stability (3) (176). The role of exon 1 in mRNA stability is complex. Differences in the mRNA half-life of c-myc with or without the first exon have been noted when analyzed in cell lines expressing the truncated or the normal gene. Viral promoter-driven c-myc genes lacking the first exon have the same short half-life as the endogenous full-length mRNA in growing fibroblasts. However the half-life of the endogenous gene transcripts decreases during growth arrest while the half-life of the transcripts that lack the first exon remains constant. Stabilizing sequences have been described in the intron 1 sequence present in the abnormal c-myc mRNAs as observed in many plamacytomas and human Burkitt's lymphomas (111).

Another very important regulation of c-myc expression is at the level of translation. Infact two translation initiation sites exist: the former is present at the 5' end of exon 2 and has all the canonical features according to the Kozak's law (87), the latter has been recently identified as a non AUG codon near the 3' end of exon 1 in c-myc. In Burkitt's lymphomas, complete removal or specific mutations of exon 1, as effected by the translocation of c-myc, correlates with suppression in the synthesis of the larger protein and thus may contribute to the oncogenic activation of c-myc (70).

3.1.2 c-myc in transformation.

The c-myc proto-oncogene has been found to be 'activated' by several different mechanisms that results in the unconstrained growth of tumor cells (34) (84) (85) (140) (142). The mechanisms of activation include proviral insertion, chromosomal

translocation and gene amplification. Significantly each of these mechanisms involves DNA rearrangements that lead to constitutive or elevated levels of c-myc expression rather than qualitative changes in the protein (159). The first demonstration that cellular proto-oncogenes could be activated in tumor cells was the finding that avian leukosis virus was integrated into the c-myc locus in viral-induced B-cell lymphomas. The retroviral insertion activates the cellular genes by an LTR enhancer activity. Meanwhile the c-myc gene was found to be located precisely at the chromosomal translocation breakpoint in murine PC's and human BL's. In both tumor types the reciprocal chromosomal translocation involves one of the three Ig locus that induce a strong activation of c-myc expression. The third mechanism of DNA rearrangements is the gene amplification. While translocations and retroviral insertions have been demonstrated only in lymphoid malignancies, this phenomenon has been described in a variety of cellular types (human colon carcinoma COLO, murine osteosarcoma SEWA, small cell lung carcinoma SCLC and HL60) where an abnormally high level of c-myc has been demonstrated (90).

Recently it has been shown that c-myc proto-oncogenes cloned in retroviral vectors are able to transform both embryonic fibroblasts and macrophages in culture and to elicit granulocytic leukemias in chickens. The c-myc dependent transformation was accompanied by a change in morphology, ability to grow in suspension, rate of proliferation, cytoskeleton structure and composition of extracellular matrix. An additional experimental strategy that has been used to define the activity of c-myc is based on cotransfection of two oncogenes into normal rat embryo

fibroblasts (REF) (125). It has been shown that SV40 promoter-linked c-myc genes and mutant ras genes could transform these cells while the ras alone could not. Because this cooperating activity can be replaced by the use of established fibroblasts or by the so-called immortalizing genes, such as adenovirus E1A, an immortalization-like function has been suggested for c-myc. However c-myc has been shown to be able to induce morphological transformation also in established cell line like Rat-1a. In these cells, transfection of c-myc, under suitable promoter, is able to induce the formation of foci, growth in soft agar and tumors in nude mice (40) (42). Furthermore a stable transfectant of the same cell line with the chimera Myc-ER has been recently constructed and shown to confer transformation of these cells only in the presence of the inducing hormone (48) (49).

3.1.3 c-myc and the control of S phase.

The possibility that c-myc may be directly involved in the regulation of DNA replication is still not clear. Cellular DNA sequences acting as putative replication origins, one of which also exhibits enhancer function, have been immunoprecipitated in complexes containing c-myc. In addition c-myc has also been found to promote extrachromosomal replication of a SV40 based vector. In contrast a variety of antibodies against human c-myc protein were unable to interfere with initiation or elongation of SV40 DNA replication in vitro.

During the last 4 years, one group has published a series of papers concluding that c-myc can bind and promote replication at the replicative origin in both SV40 and c-myc itself. In addition

this putative c-myc origin also contains a transcriptional enhancer activity suggesting that c-myc protein may regulate its own expression. There are numerous examples for trancription factors functioning at origins of replication and this work suggests that Myc might function in both transcription and replication. However these results are not completely accepted. Other attemps to show an effect of Myc on either the SV40 origin or the mouse autonomous replicating sequence (ARS) described above have failed and several laboratories have not been able either to verify that the putative c-myc ARS has origin activity, that the c-myc origin exists at that site or that c-myc protein specifically binds to the origin/enhancer.

A direct role for c-myc in the control of DNA replication comes from experiments using developing Xenopus laevis embryos. In the mature oocyte, c-myc is one of the stored maternal proteins and resides in the cytoplasm until fertilization when it is rapidly translocated to the nucleus. The redistribution of the myc protein coincides with the zygote entering 12 synchronous cell cleavages which occur within 30' intervals in the absence of any transcriptional activity. Injection of myc-specific antibodies blocked these rapid cell cleavages while coinjection of the same antibodies preparation together with its peptide antigen had no effect. The import and turnover of Myc in these nuclei strongly suggest a functional role for c-myc that could be related to the activation of embryonic DNA replication since the embryos are transcriptionally inactive.

If the hypothesis that c-myc is directly involved in DNA replication at the biochemical level is still debatable, a wealth of evidence has defined a role of c-myc in the capacity of a cell

to enter the S phase either by inhibiting or enhancing its expression. Although some of the earliest experiments showed that introduction of antibodies against Myc appeared to inhibit DNA synthesis, later work demonstrated that these results were artifactual. On the contrary, some antisense oligonucleotides complementary to c-myc mRNA were able to inhibit both the proliferation of human leukemia cell line HL-60 and S Phase entry of human resting T lymphocyte activated by mitogenic signals (72). In the last work the analysis of the expression of marker genes specific for defined stages during G0/S transition suggest that the inhibition by antisense oligonucleotide is at the level of G1/S progression and not in the G0/G1 transition.

Ectopic overexpression of c-myc has given different effects according to different systems, conditions and operators. While the microinjection of resting NIH3T3 cells with E1A was able to induce the entry into S phase, the overexpression of c-myc had the same effect as the vector alone (155). At the same time two other papers suggested that c-myc indeed was able to induce the entry in S phase. Armelin et al. produced BALB/c3T3 stable transfectant subclones containing the c-myc gene in which expression could be induced by the addition of steroid to the cell culture. Once these subclones were density-arrested and PDGF deprived, the addition of c-myc via steroids addition leads to an activation of the entry in S phase (28% of 3H-Thymidine positive cells against 4% in not steroid treated cells).

Meantime Kaczmareck et al. showed that the c-myc protein, once microinjected into the nuclei of quiescent Swiss 3T3 cells, cooperated with platelet-poor plasma (PPP) in the stimulation of cellular DNA synthesis. This experiment was based on the

observation that PDGF, by itself, cannot stimulate DNA synthesis but this occurs if cells are treated with PPP after exposure to PDGF. PPP, by itself, has no effect. Infact PDGF makes the cells 'competent' and PPP allows their progression into the S phase (progression factor). Swiss 3T3 cells were made quiescent via contact-inhibition and subsequent treatment with 1% FCS. Microinjection of the c-myc protein not followed by addition of PPP had no effects on the percentage of cells labelled by [3H] thymidine; on the contrary cells treated with PPP after microinjection were considerably induced in S phase: 19.7% against 1.5% of not microinjected cells. This increase was roughly half of the total effect of the addition of 20% FCS.

Recently a chimera composed of the hormone-binding domain of the human estrogen receptor fused with the C-terminus of the MYC protein (previously shown to give estrogen dependance of the transformed phenotype in established rat cells), was used to study the effects of c-myc induced expression on the entry in S phase. Stable transfectants of the chimeric gene were established in Rat-1a and BALB/c3T3 cells. Estrogen mediated activation of c-myc expression in high density arrested Rat-1a cells was able to induce 30/40% of cells to enter S phase, a level comparable to the mitogenic effects of fresh serum. Similar results were also obtained in BALB/c3T3 cells.

Overexpression of c-myc protein in growing Swiss 3T3 cells also results in a dramatic shortening of the G1 phase of the cell cycle.

3.1.4 c-myc protein structure.

Secondary structure predictions based on amino acid sequence information suggest that c-myc is composed of an α-helix/β-sheet domain (aa 1-203) and a predominantly α-helical domain at the carboxyl end (aa 238-439), separated by a less structured hinge region (aa 204-237). The primary sequence presents several interesting aspects. At first 4 consensus sequences for phosphorylation for different kinases are present and all of them have been confirmed by an *in vivo* analysis. Phosphate is present in both viral and cellular myc proteins and phosphamino acid analysis of Myc isolated by immunoprecipitation indicate that the phosphate is covalently bound to Serine and Threonine residues. Ser 62 is a putative site for $p34^{cdc2}$, while Thr 58 presents a target for a not identified kinase: this last site is mutated in several retroviral v-myc genes. The two other phosphorylation sites have been shown to be substrates for casein kinase II. One site is localized beetwen aa 240-262 in human c-myc and aa 222-238 in the avian protein. The other site is localized within aa 342-357 (103).

The aminoterminal c-myc region contains a high relative number of proline and glutammic acidic residues that are usually present in the activator domains of transcription factors (83). Region A (1-41) includes a 22 aa segment with 32% glutammic acid residues while region B (41-104) includes a 21 aa region presenting a 33% proline content. To establish if Myc might regulate transcription in vivo, fusion proteins between segments of human c-myc and the DNA binding domain of the yeast transcriptional activator GAL4 were constructed and assayed for

their ability to activate transcription from a reporter gene linked to GAL4 binding sites. Three independent activation regions were identified and localized within aa 1-143. These results demonstrate that domains of the c-myc protein can function in transriptional regulation of a model system and suggest that alterations of Myc transcriptional regulatory function may be involved in transformation. More recent studies have tried to analyze the interaction of cellular proteins with this c-myc domain. Region 1-206 has been shown to bind the retinoblastoma protein and the TATA-binding factor TFIID *in vitro*, however these interactions have been shown to occur only in presence of high concentrations of one of the subunits and have never been demonstrated *in vivo* until now.

The carboxylterminal region of the myc protein has attracted particular interest since it contains three structural motifs that were first identified in several DNA binding proteins. Two of these structures, present in the c-myc carboxy-terminus region, the Leucine Zipper (LZ) and the Helix Loop Helix (HLH) can promote protein-protein dimerization while the third structure, the basic domain (BR), can mediate sequence specific DNA binding. In human c-myc, BR is contained within aminoacid 354-369, the HLH motif from 370 to 401 and the LZ from 406 to the C-terminus. The LZ is defined as a coiled-coil of parallel amphipatic α-helices, each helix containing an heptad repeat of leucine residues along its hydrophobic dimerization interface. The LZ has been found in transcription factors like C/EBPα, c-fos and c-jun. The HLH region is characterized by two predicted amphipatic α-helices containing hydrophobic residues at every third or fourth position. The helices are separated by a region which usually

contains one or more helix breaking residues and has been modelled as a loop. This structure has been found in MyoD, the immunoglobulin enhancer binding protein E12 and E27 and their Drosophila homologue daughterless as well as the proteins of the Drosophila achaete-scute complex.

The presence of these motifs, usually involved in protein-protein interactions, have suggested both the existence of homodimers and the possibility for heterodimer formation with other cellular partners. Although at high concentrations c-myc protein has been shown to form dimers and tetramers, at concentrations approaching the physiological range c-myc oligomerization appears to be relatively inefficient. The sequence specific DNA binding properties of c-myc have been challenged by two different approaches. High concentrations of bacterially expressed gluthatione-S-transferase Myc chimeric proteins were used to drive oligomerization and DNA association with a generate pool of partially randomized oligonucleotides. The subsequent amplification and new incubation with the purified proteins led to enrichment for the oligonucleotide containing the site recognized by c-myc (17). Meanwhile, another group used an in vitro synthesyzed E12/c-myc chimeric protein containing the c-myc basic region and the dimerization domain of the E12 HLH to test different E box sequences by Band Shift analysis (132). Both approaches lead to the conclusion that NNCACGTGNN is the major DNA binding site for c-myc protein. The same two groups were also able to clone the myc cellular protein that interacts via HLH/LZ domains. Blackwood et al. screened a cDNA expression library with iodinated gluthathione-S-transferase-B/HLH/LZ myc fusion protein (18). They were able to clone Max, whose product

was shown to form heterodymers both in vitro and in vivo. These heterodimers were able to bind DNA in a sequence specific manner. Prendergast et al. cloned the murine homologue of Max, Myn, using an RT-PCR primed with degenerated oligos within the B/HLH/LZ region (134).

The transcriptional regulatory function of Myc and Max has been dissected in yeast and mammalian cells (6) (19) (89) (105). Since there is no evidence for Myc and Max homologues in S. cerevisiae, yeast cells were used as an in vivo system to show that the myc protein is indeed a sequence specific transcriptional activator whose DNA binding is strictly dependent on dimerization with Max. The transactivation effect is mediated by the aminoterminal domain of Myc. Infact Max homodimers are able to bind to the same DNA sequence as Myc/Max heterodimers but they fail to transactivate a reporter gene; moreover Max has a higher affinity for Myc than for itself. In mammalian cells it has been shown that Myc overexpression activates, while Max overexpression represses, transcription of a reporter gene. Repression requires the DNA-binding domain of Max whereas relief of repression requires the dimerization and transcriptional activity of Myc. Max expression is not significantly affected by changes in the state of cell growth: it is present in quiescent as well as in growing cells and it is highly stable. Moreover its expression does not appear to be influenced by c-myc overexpression. Since *in vitro* binding studies demontrate that max can form homodimers, quiescent cells would contain a major fraction of Max/Max homodimers while serum-stimulated and growing cells contain Myc/Max complexes.

3.1.5 c-myc functional domains.

The identification of functional domains of the c-myc protein have defined the structural basis for its activity. This kind of analysis has been performed by constracting a series of mutants of c-myc protein and analyzing the ability to confer the same biological effect as the wild-type in different assays. The first dissection of the functional properties of c-myc protein has been performed by Stone et al. They analyzed sequences involved both in myc ability, in cooperation with EJ-ras gene, to transform established cell line. Some of the mutants of the c-myc gene cotransform REC cells with decreased efficiency. However the cells transformed by these mutated c-myc genes and EJ-ras were indistinguishable by several different criteria (morphology, ability to form foci and tumorigenicity). Both deletion and insertion mutations in regions 106-143 and 318-433 affect or abolish activity indicating that they contain some features that are essential for cotransformation. The region 1-103 is important but only larger deletions severely affect the activity and insertion or smaller deletions present lower or no effects. Finally the region 143-318 appears to be largely dispensable for cotranformation. These findings were consistent with results previously published on domains of MC29 gag-myc fusion protein that are important for chicken fibroblast transforming activity. Given that Rat-1a cells undergo morphological transformation after infection with a retrovirus carrying wt c-myc cDNA, some c-myc mutants were cloned into a retroviral vector and packaged in order to infect Rat-1a cells. This new assay shows the role of the three different domains to partecipate in the transformation

of an established cell line in the absence of a second oncogene. Both region 106-143 and region 318-433 resulted to be absolutely required for a fully functional c-myc protein.

Ectopic c-myc expression is also able to induce the suppression of the endogenous gene expression in different cells and it has been proposed that this property could be involved in the transformation capacity of this gene. The mutational analysis of the regions involved in the autosuppression has been performed and has shown that regions 106-143 and 367-433 are the only regions that, once deleted, were able to affect this phenomenon; while deletion of the first 100 aa or within region 145-367 has no effects. These results were confirmed in the analysis of the avian c-myc protein where the C-terminus was shown to be involved both in transformation and in autoregulation.

3.2 The quiescent state.

While growth factor availability allows cells to undergo a series of asinchronous divisions, serum deprivation or cell to cell contact inhibition allows cells to enter into a resting state referred to as G0.

The relationship between growth arrest induced by these two different conditions have been studied for many years. The role of cell to cell contact in a dish culture has been initially analyzed referring to inhibition of movement. Later this concept has been extended to the inhibition of growth.

When a confluent culture of 3T3 cells is wounded, new growth occurs at the margins of the wound. This indicates that suppression of growth in the intact confluent sheet is under local

control, a phenomenon known as 'topoinhibition' and this reflects the direct role of the cell to cell contact in the establishment of this growth arrest state. However this phenomenon was alternatively explained as the consequence of the local depletion of growth factors. This second hypothesis has been substained by inducing a gentle laminar flow of medium across the wound of a contact inhibited cell culture. In these conditions growth remains suppressed at the upstream margin of the wound despite the reduced level of intercellular contact (2) (47) (78) (158) (165).

Other experimental data supported a main role of growth factors in density dependent inhibited cell culture. If cells are grown in medium containing different serum concentrations, arrest is induced at different cell densities. Moreover the medium of a contact inhibited cell culture is not able to support growth in sparse cell culture suggesting depletion of soluble growth factors.

At the molecular level, no differences in gene expression between these two quiescent states have resulted so far.

Recently a Tyrosine phosphatase activity has been discovered to be induced in contact inhibited but not in serum starved growth arrested cells (120).

Independently from how this state is induced, a growth arrested cell differs from a cycling cell in many features.

Pioneeristic studies had focussed on peculiar biochemical properties of quiescent cells such as decreased size, lower rate of macromolecular synthesis, monosomal structure of ribosomes(121) (122).

More recently RNAs and proteins that are specifically present in growth arrested cells have been identified in different

systems (13) (14) (22) (55). The first analyzed quiescent specific marker was Statin. It is a 57 Kd protein located in the nucleus of growth arrested and senescent human fibroblasts and absent in young and cycling cells (169).

Serum deprivation or density dependent growth inhibition in cultured murine NIH3T3 are both able to induce the expression of a particular set of genes called gas (growth arrest specific). When growth arrested cells are reintroduced into cell cycle, expression of gas genes is rapidly down-regulated (146) (144). Gas genes seems to be strictly associated with the G0 and their downregulation could rapresent a prerequisite for entering a new cycle. The study of gas gene expression during G0/G1 transition has also suggested a primary role of postranscriptional control mechanisms in their regulation as well as their dependancy on an active transcription/translation machinery (30) (106). Moreover gas gene products have been localized in different subcellular compartments suggesting that the arrested state is really a different cellular 'scenario'.

Gas2 gene product has been shown to be present at the level of the cell border where it colocalizes with the microfilament network system; it is also detectable, albeit at lower density, along the stress fibers. When it is microinjected in growing NIH3T3 cells, it localizes in the microvillar apparatus suggesting that this protein is a component of the cortical actin filament system. The mitogenic activation of quiescent cells provokes an immediate phopshorylation of Gas2 protein (23).

Gas3 and Gas1 code for membrane proteins.

In vivo Gas3 is highly expressed in differentiated quiescent Schwann cells in the peripheral nervous system (PNS) and,

following sciatic nerve injury, is rapidly down-regulated (106) (144). After nerve injury infact Schwann cell proliferation is resumed: this rapresents the first example of an *in vivo* regulation of a gas gene. Gas3 is a major component of PNS myelin and this indicates that it could be a differentiation specific gene involved in myelin formation. However its mRNA is expressed in a regulated manner in fibroblasts and is also highly expressed in other non neuronal tissues. Moreover it appears to be down-regulated in chemically induced tumors of the murine lung (137). It has been recently shown that two different point mutations in two membrane domains of gas3 seem to be responsible for the *tr* (*trembler*) phenotype in mice (160). This pathology is characterized by both severe hypomyelination of PNS and continuous Schwann cell proliferation. Moreover the gas3 human homologue has been shown to be involved in the Charcot-Marie-Tooth disease type 1A (CMT1A) which is an autosomal dominant peripheral neurophaty (123).

Gas1 is the only gas gene regulated at the transcriptional level in fibroblasts. Ectopic expression of gas1 in proliferating cells has been shown to inhibit the S phase entry in NIH3T3 cells and in a panel of transformed cells. This inhibition does not alter the 'immediate early' response. Gas1 is not able to act as a growth suppressor gene in SV40 transformed fibroblasts, thus suggesting that the block of the S phase entry is possibly transduced by LT antigen binding proteins as Rb and p53 (45).

Gas6 codes for a secreted protein belonging to the vitamin K dependent protein family. It presents 43% homology (aa identity) with protein S, which is prevalently expressed in liver, while gas6 is expressed in most cellular types. This suggests that even

though gas6 and protein S show similar structural motif, reflecting a common evolutionary origin, they might have different functions (144).

No informations has been collected on Gas4 and Gas5 gene products. However while Gas5 mRNA accumulation in Go is controlled at the postranscriptional level, in differentiating cells (Friend erythroleukemia cells) Gas5 seems to be regulated at the transcriptional level (32).

The absence of any nuclear located products among the various gas genes can suggest that 'early' gas genes have still to be identified. It is possible that the analysis of gas1 transcriptional regulatory elements can lead backward into the early events that control the growth inhibitory program. This has infact two requirements in order to be operative: 1. a lower active level of positive elements in growing cells 2. a higher functional level of the negative elements. The transition between an established growing compartment to the unestablished arrested state might thus 'develop' through a growth shut down transition in which a cell cycle block reversible and compatible with cell survival is induced. Since cells seem to need signals from other cells to proliferate and neutralize the growth inhibitory pathway, similarly they would need signals to survive and neutralize the programmed cell death (see above). Survival control elements are required to escape the constitutive apoptotic pathway and they are necessary for the maintenance of the arrested state. Survival and differentiation factors/elements in this model do not bear separable information since they are both required for life of a not growing cell.

3.3 Programmed cell death or apoptosis.

The cytological characteristics of normal cell death are generally different from those seen in acute pathological cell death (175). In acute pathological cell death, which results from cell injury, the cell tends to swell and lyse and cell's contents spill into the extracellular space inducing an inflammatory response. This process is named 'necrosis'. In normal cell death, on the contrary, the nucleus and cytoplasm shrink and often become fragmented, while cells are rapidly phagosytosed by their neighbours or by macrophages. Since cell contents do not leak into the extracellular space, there is no inflammation. This process is called 'apoptosis'. Because dead cells are rapidly removed without inflammation, even large-scale normal cell death is often histologically undetectable and this could be the explanation why this process has been so underconsidered until now.

Infact there is forthcoming evidence that apoptosis is indeed a normal and largely used regulatory step during cell proliferation/differentiation control and body development. There are several examples: the tadpole tail during metamorphosis, the Mullerian ducts that are needed in only one sex, cells during the process of increasing the body or in regions between developing digits in amniotes, lymphocytes that have failed to produce functional antigen-specific receptors, neurons that are produced in large excess during development before selection.

Thus programmed-cell death process is tightly regulated by hormones and growth factors and by cell to cell communications/interactions. The regulatory signal could be either a stimulus, such as glucocorticoid hormones for immature

thymocytes, or the disappearance of a stimulus, such as interleukin-2 withdrawal from mature T lymphocytes or the removal of colony stimulating factors for hemopoietic precursors. In these cases, the presence of the relevant factor or of the intercellular interaction can effectively suppress cell death.

Programmed cell death of lymphocyte, prostatic cells and many other cell type involves rapid DNA fragmentation into 180 bp multimers by a Ca^{++} dependent endonuclease. However this phenomenon might not be universal because in Nematodes at least some of the DNA degradation is caused by a Ca^{++} independent endonuclease controlled by the nuc-1 gene. To identify proteins that cause cell death, several researchers are studying cDNAs from genes that are highly expressed in dying cells. Such work has demonstrated that expression of a gene, SGP-2 that code for a secreted protein, is strongly induced in dying cells. During similar studies of mRNAs induced in dying thymocites, a β-galactoside binding protein and a zinc-finger protein have been identified but whether these proteins play an active role in cell death is still unknown (118).

The Nematode *Chaenorabditis elegans*, whose cellular anatomy and cell lineages are precisely defined, has provided the most powerful model system for analyzing the genetics of programmed cell death. Of the 1090 somatic cells found during the development of an adult hermaphrodite, 131 die, each with morphogenetic features resembling apoptosis (74). Several genes have been identified whose expression marks the cells that have to die (egl-1, ces-1, ces-2) as well as genes involved in active cell death itself (ced-3, ced-4 and ced-9), in the phagocytosis

(ced-2, ced-5, ced-6, ced-7, ced-8, ced-10) and in the disposal of the dead cells (nuc-1). The genes ced-3 and ced-4 have resulted particularly interesting. They have a role very early in the process in a cell autonomous manner and in the absence of these two functional genes, cells fail to die and differentiate. On the contrary the ced-9 gene product is able to suppress cell death. A mutation causing a gain of ced-9 function prevents almost all the apoptotic process, while mutations that inactivate this gene cause a large number of ectopic cell death and are lethal to the animal: mutations in ced-3 and ced-4 prevent these deaths and the associated lethality. Thus ced-9 probably regulates programmed cell death by blocking the activities of ced-3 and ced-4. All ced-9 mutations show strong maternal effects indicating that the ced-9 gene product is contributed by the mother to the eggs.

In the mammalian system, a functional analogue of ced-9 is bcl-2. This gene was identified in the break site of many t(14:18) chromosomal translocations which are often found in human B-cell lymphomas. These translocations cause both bcl-2 mRNA and protein to be expressed at high levels. There are several evidences for the involvement of bcl-2 in the suppression of the apoptotic process:

1. haemopoietic cells undergo programmed cell death when deprived of certain growth factors but such deaths are delayed or prevented by bcl-2 expression

2. bcl-2 expression can prevent B- and T- lymphoblasts from undergoing programmed death induced by stress

3. mice carrying a transgene causing high levels of bcl-2 expression in the immunosystem shows extended survival of B and T cells.

Bcl-2 gene encodes a membrane protein that appears to be associated with the inner membrane of mitochondria (50). Since intracellular Ca^{++} levels play an important role in lymphocyte death, one can speculate that bcl-2 is involved in the balance beetwen intracellular and mithocondrial Ca^{++} amounts.

Another important aspect of the regulation of apoptosis is the ability of some viruses to actively repress the programmed death of infected cells. The Epstein-Barr virus effects on the mammalian B cells are mediated by the latent membrane protein LMP-1 whose expression is correlated with the level of bcl-2 protein, suggesting that LMP-1 might prevent cell death by inducing bcl-2 (73).

Although the regulation of cell numbers is usually considered mainly in terms of control of cell proliferation, these observations suggest that control of cell survival can play an important role even in mature animals. It is possible that hormone and insulin-like growth factors stimulate growth at least partially in this way; infact for some types of cells in culture, insulin-like growth factor promote cell survival rather than cellular proliferation. Moreover competition for survival signals could be one of the mechanisms in order to have a perfectly balanced cell turnover so that the number of cells produced by division is exactly matched by the number of cells that die.

Recently it has been shown that in some circumstances expression of c-myc is highly related to the induction of

programmed cell death (52). Rat-1a cells containing a normal human c-myc gene driven by a constitutive promoter were coltivated at various serum concentrations and the number of living cells was counted at daily intervals. Growth curves of all the cells appeared very similar, irrespective of whether or not the cells constitutively expressed active c-myc and with an average cell number strictly dependent on the presence/absence of growth factors. These results suggest that constitutive c-myc expression has no effects on the ability of Rat-1a cells to slow their growth in low serum levels. However analyzing the Rat-1a/myc cells in low serum for DNA content and BrdU incorporation by flow cytometry, the existence of a complete block of growth emerged in cells constitutively expressing c-myc. This behaviour was fully explained by the loss of cells in serum deprived cultured and it was demonstrated that this phenomenon was due to apoptosis. The same results were obtained by inducing the expression of the chimera c-myc-estrogen receptor in stable Rat-1a transfectants, in REF cells, in Swiss and NIH3T3 cells. If the regions 7-91, 106-143 and 371-433 are mutated, the induction of apoptosis was suppressed while deletion of the 145-317 region had no observable effect.

The involvement of c-myc in apoptosis was also shown in other two systems. In the murine IL-3 dependent myeloid cell line 32D, downregulation of c-myc expression is an immediate response to IL-3 deprivation (10). This is followed by an accumulation of cells in the G1 phase of the cell cycle. However clones of 32D cells with an ectopic c-myc gene constitutively expressed fail to arrest and rapidly activate apoptosis. Immature T cells and some T cell hybridomas undergo apoptotic cell death

when activated through the T cell receptor complex, a process that is probably related to antigen induced negative selection of developing T cells (150). Antisense oligonucleotides corresponding to c-myc block the constitutive expression of c-myc protein in T cell hybridomas and interfere with all aspects of activation-induced apoptosis without affecting lymphokine production in these cells. All these data suggest the hypothesis that Myc is able to activate the programmed cell death pathway. Moreover the absolute indipendence of the activation of apoptosis from the point of the cell cycle can suggest that the cellular ability to undergo apoptosis is governed by a yet unidentified stochastic fact. Moreover, in the case of serum starved Rat-1a/myc cells those cells not dying continue to proceed through the cell cyle, consistent with the established mitogen properties of c-myc as well as when Rat-1a/ myc-ER cells 48 hours in low serum are treated with estradiol, many cells survive the first wave of apoptosis, enter S phase after about 12 hours and subsequently divide. Within this population, however, apoptosis continues and the fate of the culture is ultimately determined by the relative rates of myc-induced death and myc-induced proliferation. One can hypothize that the proliferation advantage due to c-myc activation is essential for carcinogenesis. However since both the proliferation and apoptosis inducing features of Myc are highly coupled, it is impossible to select for one without also selecting for the other. If this is correct, there are three mechanisms that might allow a cell with deregulated c-myc to survive: acquisition of a second mutation in a second proto-oncogene (cooperation), continuous stimulation by mutagenic hormones/growth factors, a lesion in the programmed cell death

pathway. This last possibility is particularly intriguing: the bcl-2 gene is able to cooperate with c-myc in the establishment of human lymphoid malignancies. This ability could reflect an active role of bcl-2 in the suppression of the apoptosis pathway activated by the constitutive overexpression of c-myc. Recently this cooperation has been well documented in a series of *in vitro* experiments were apoptotic cell death induced by an ectopic expression of c-myc can be blocked by bcl-2 (16).

3.4 Differentiation.

In Vertebrate development, a prominent feature of several cell lineages is the coupling of cell cycle regulation with terminal differentiation. In eukaryotic cells this process involves a controlled progression of cells through successive pre-differentiation stages and growth inhibition. The study of the relationship between cell proliferation and differentiation have been approached using in vitro models. The mechanisms by which this predifferentiation state can be achieved are dependent upon the integration of positive and negative effects of cell proliferation control with differentiating agents. It has been shown that in the majority of cells the ectopic inhibition of growth arrest has large consequences on the differentiation process.

In most of the systems analyzed the c-myc gene has a higher level of expression in proliferating cells than in differentiated cells even if some exceptions have been reported in keratynocites and F9 embrionic cell line (116). Deregulated and continued expression of c-myc blocks terminal differentition

induced by IL-6 or LIF at an intermediate stage in the progression from mature blasts to mature macrophages, precisely at the point when c-myc is normally suppressed, leading to intermediate-stage myeloid cells which continue to proliferate (77). In the HL-60 promyelocytic leukemia cells, cells are still able to differentiate either to the granulocytic pathway when treated with DMSO/retinoic acid or along the monocytic pathways when treated with phorbol esters or vitamin D analogs. The direct inhibition of c-myc expression with the anti-myc antisense oligonucleotide was associated with a decreased cell growth rate and an induction of myeloid differentiation (79). High constitutive expression of c-myc blocks the differentiation of mouse erythroleukemia cells into cells that resemble mature erythrocytes, while expression of its antisense accelerates this process (136) (153) (154).

Two systems were particularly well studied in terms both of positive and negative regulations of cell proliferation.

During skeletal muscle development, multipotential precursor cells progress through a determined myoblast stage before differentiating to become myocites. While precursors and myoblasts proliferate extensively, withdrawal from the cell cycle is required for differentiation (4).

During myogenesis, c-myc is expressed in proliferating myoblasts but its mRNA and protein levels drop substantially when these cells withdraw from the cell cycle and differentiate; moreover differentiation-defective derivates of some myoblast cell lines fail to down-regulate c-myc expression. It has been shown that ectopic c-myc expression can interfere with myoblast differentiation in quail myogenic cells and that cotransformation

assays with the MyoD gene in NIH3T3 cells demonstrated the c-myc ability to suppress the MyoD-induced differentiation. However things are not so simple: infact the c-myc gene is indeed inducible in both biochemically and terminally differentiated myotubes treated with mitogens and its transient induction does not suppress the differentiated phenotype. This result would thus indicate that irreversible suppression of c-myc transcription is not required for terminal myogenic differentiation in this cell line and that re-expression of c-myc is not sufficient to affect transcription of muscle-specific genes. However c-myc gene down-regulation is indeed a necessary event for terminal differentiation but probably it must occur just transiently at a specific point during the differentiation program (110).

Coincident with the inhibition of growth-inducing stimuli, at the beginning of the process genes able to induce the differentiation are switched on. In this system, expression of MyoD (Muscle Determination Gene) induces the formation of myoblasts. It has been shown that this transition is accompanied by the exit from the cell cycle. Infact MyoD is able to cause growth arrest when expressed in a variety of cells independently from its involvement in differentiation. The MyoD product has been shown to associate with another protein called Id via the helix-loop-helix domain. This interaction regulates the activity of MyoD by inhibiting its DNA binding activity.

Another very well characterized system is the 3T3-L1 preadypocyte cell line. Confluent monolayer of these cells lost their responsiveness to high concentration of serum after a 48 hours treatment with differentiation inducers. It is likely that such cells were initiating the commitment process in which they

irreversibly withdraw from the cell cycle and terminally differentiate without further expoure to inducers. In contrast cells expressing high constitutive levels of myc mRNA re-entered S phase when exposed to 30% serum after treatment with differentiation inducers. Such cells were not capable of withdrawing from the cell cycle and thus failed to terminally differentiate. These findings indicate that normal 3T3-L1 cells enter a distinct state in G0/G1 after treatment with differentiation inducers where cells expressing high levels of myc RNA are precluded from entering (57) (58). A series of mutants of human c-myc gene were stably transfected into the 3T3-L1 preadypocite cell line and assayed for their capacity to block differentiation measuring the induction of glucophosphate dehydrogenase. The deletion of the N-terminus (7-91), of the region 106-143 and of the C-terminus (371-433) inhibits completely the effects seen for the ectopic expression of wt c-myc. Moreover deletion in 145-319 region is able to completely shut off the differentiation inhibiting capacity. The C-terminus region has been also shown to be involved in the inhibition of erythroleukemia differentiation.

Another gene, named c-EBPα, has been shown to be involved in this process. When overexpressed, it is able to induce both differentiation and growth arrest. It codes for a DNA binding protein containing the BR and LZ domains and appears to regulate the expression of a variety of genes that control energy metabolism. However this gene doesn't seem to be involved only in the differentiation process of adypocites but also of hepatocytes, where its expression is very high in the non growing

and actively metabolizing normal adult tissue and very low in rapidly growing hepatoma cells.

3.5 From quiescence to the growing state.

A quiescent cell, once activated by growth factors addition, is able to reenter the cell cycle and start DNA replication 12 hours later.

The ways in which a cell starts to replicate its own DNA from an arrested state has been analyzed in two perspective.

In the first, G1 has been divided into subphases depending on the effects of limiting growth factors, nutrients or inhibitors as measured by time to reach S Phase after the block is removed.

In the latter attention was focussed on the molecular aspects of growth factor/cellular receptor interactions and the transducing pathways that different growth factors are able to activate.

3.5.1 From ligand/receptor interaction to second messengers.

A large group of growth and differentiation factors act by binding to and activating surface receptors with intrinsic protein Tyrosine kinase activity (143) (124). All receptor Tyrosine kinases are composed of three major domains: an extracellular domain, a single membrane-spanning and a cytoplasmic domains. The extracellular domain is responsible for ligand binding and transmission of the biological signal to the cytoplasmic domain, whose role is to transmit the biological signal to intracellular

target proteins. The cytoplasmic domain contains, in addition to the catalytic protein Tyrosine kinase, distinct regulatory sequences with Tyrosine, Serine and Threonine phosphorylation sites. The Tyrosine kinase activity of growth factor receptors is essential for the signal transduction pathway of mitogenesis, transformation and cell proliferation. Following ligand binding, all known growth factor receptors appear to undergo dimerization, which is responsible for the activation of the intrinsic protein Tyrosine kinase activity. Moreover the dimerization induces the autophosphorylation that occurs in regions that represent specific binding sites for cytoplasmic target proteins involved in transmission of the biological signal. The association between the Tyrosine-phosphorylated regions in growth factor receptors and the signalling protein occurs in a conserved region of approximately 100 aa, termed src homology 2 domain (SH2). These domains represent the recognition motif for specific Tyrosine-phosphorylated peptidic sequences. On the basis of their primary structures, it is possible to divide these proteins in two main classes. Type I defines proteins that contain, in addition to the SH2, distinct enzymatic activity, such as phospholipase (PLC-γ), Tyrosine kinase (c-src) and GDP-GTP exchange functions (c-vav). The second class is composed of only SH2 and SH3 domains where the SH3 is another well conserved domain of 50 aa. These proteins are thought to function as adaptors or regulatory components of specific catalytic subunits (c-crk, c-nck, GRB2/sem-5). The identification of Tyrosine autophosphorylation sites in growth factor receptors combined with functional analysis of mutated regions in transformed cells has enabled the assigment of specific binding sites for different

signal proteins. Thus the activity of a Tyrosine kinase receptor equals the sum of the activators of the signalling proteins that interact with its Tyrosine phosphorylated form.

SH3 domains are also found independently of SH2 domains. They have been identified in the products of several genes in budding and fission yeasts where no SH2 domain have been identified so far. It has been recently hypothized that SH3 domains are involved in the control of small Ras/like guanine nucleotide-binding (G) proteins. Infact a mammalian cDNA cloned through its property of binding the SH3 domain resulted to be related to a group of proteins with GTPase-activating activity. These proteins are often associated or able to regulate the cytoskeleton. Thus in the context of SH2-containing protein, SH3 domain may link Tyrosine kinases to pathways controlled by small G proteins.

A lot of key enzymes involved in growth control have been identified as PDGF receptor's substrates: phospholipase C-γ (PLC-γ), phosphatidylinositol 3 Kinase (PI-3K), Ras guanosine triphosphatase (GTPase) activating protein (GAP), src and src-like Tyrosine kinases, c-raf proto-oncogene. These results were compared to the data collected for other growth factors like EGF, FGF and insulin or tumor promoters like TPA (26) (36).

PLC-γ is one of several PLC isoforms and it hydrolyses phosphatidylinositol 4,5-biphosphate generating two second messengers: inositol triphosphate and diacylglycerol. These provoke the release of stored intracellular calcium and the activation of protein kinase C (PKC).

PI-3K, which phosphorylates the inositol ring of phosphatidyl-inositol, becomes physically associated with a

number of activated Tyrosine kinases. The protein is formed by a dimer of a 110 Kd and 85 Kd subunits.

GAP regulates the function of the Ras protein. Ras is a critical component of intracellular mitogenic signalling pathways. Infact microinjection of oncogenically activated Ras into NIH3T3 cells is able to induce DNA synthesis. GAP acts as a negative regulator of Ras function. Infact mutation that cause oncogenic activation of ras lead to accumulation of Ras bound to GTP, the active form of the molecule. These mutations block the ability of GAP to promote conversion of Ras to its inactive, GDP-bound form. GAP may also function in a complex with Ras as an effector of its downstream signalling functions. Stimulation of receptors results in physical interaction of GAP with the receptor kinase. Tyrosine phosphorylated GAP is also found to be associated with at least two other tyrosine phosphorylated proteins p62 and p190 that modulate Ras function. Stimulation of cells with PDGF leads to an increase in the amount of GTP-bound Ras, suggesting that the Tyrosine phosphorylation of GAP associated proteins interrupts its inhibition of Ras function.

The src and structurally related proteins are nonreceptor Tyrosine kinases that are rapidly activated in cells stimulated with growth factors.

The raf proto-oncogene is a Serine-Threonine kinase that is activated by a PKC-independent mechanism.

3.5.2 Changes in gene expression.

When the signal is fully transduced at the nuclear level, a major change in the pattern of cellular gene expression occurs.

More than 80 genes are induced at the transcriptional level and independently from an active protein synthesis after addition of 20% FCS to quiescent cells (5) (75) (33) (63) (93). These genes are named 'primary response' or 'immediate early' genes. Most of these genes are not present in growing cells suggesting the specificity of G0/G1 transition gene expression; moreover some of these genes like c-fos, c-jun and c-myc are proto-oncogenes confirming the central role of these genes in growth control (183).

Many differential screenings of cDNA libraries have been used to identify genes induced by a variety of agents such as serum, PDGF, TPA, EGF and so on; they include transcriptional modulators, structural proteins, cytokines and proteins of a yet unknown function.

Few studies have identified the mRNAs and proteins that appear in mid or late G1; among these ras seems to be involved in the induction of S Phase. However two different screenings have been recently done in order to clone genes expressed late after activation of not proliferating cells.

One screening has been performed in bone marrow-derived mouse macrophages that, once deprived for CSF, arrest their growth in early G1 but are then able to reenter cell cycle after growth factor readdition. Among many clones, three of them resulted to code for cyclins.

Once more this result shows the strict interdependence of cell proliferation and cell cycle controls particularly referring to the necessity of a continuous cross-talk between these two networks. A cyclin has been also cloned during screening for DER

(Delayed Early Response) genes that are induced after some hours from the serum addition in BALB/c 3T3 cells (92).

Cyclins and cdks could thus represent points where many signals can be transduced into only one final message that allow or prevent passage through START and committment to the S phase (108).

3.6 Negative regulators of cell growth.

The microinjection of RNAs extracted from quiescent cells has shown growth inhibitory capacity on G1 progression in many systems. Poly(A)+ RNA from normal rat liver can inhibit DNA synthesis in human diploid fibroblasts and this inhibitory activity was not found in the poly(A)+ RNA from regenerating liver (99) (100). These mRNAs were not able to inhibit if injected less than 3 hours before the S phase entry. The growth inhibitory property has been shown also in human liver mRNAs. Poly(A)+ RNA purified from resting T cells was able to block DNA synthesis in both synchronized human fibroblasts and Hela cells, while mRNA isolated from actively growing (72 hours after mitogenic stimulation) T cells lacks this capacity (128). In this system DNA synthesis was not completely inhibited but only delayed and both types of recipient cells were able to recover from the inhibition. The inhibitory capacity of the microinjected mRNA was lost both when the donor cells were growth stimulated or when the recipient cells entered DNA replication.

Polyadenilated mRNA isolated from senescent human diploid fibroblasts (HDF) were also able to inhibit DNA synthesis in

proliferation competent cells after microinjection whereas RNA from young HDF had no inhibitory effect (109).

Even the addition of isolated plasma membranes or of membrane proteins to cultured cells reduced the growth state in a concentration-dependent manner (173).

Different methodological approaches have been used to isolate genes with an antiproliferative activity and several overlapping families have been defined.

Many studies with heterodykarions between senescent and quiescent cells on the one hand and actively replicating normal or transformed cells on the other, provided evidence that the replicative potential of cells may be recessive and inhibition of DNA replication can be observed in these hybrids (107) (172). These hybrid cells had unstable karyotypes and frequently lost chromosomes. In this case the hybrid cells would often revert back to a tumorigenic state. Correlation of this reversion to tumorigenicity with the loss of specific normal chromosomes led to the conclusion that these chromosomes carry genes that can normalize the growth program of cancer cells. A second clue to the existence of tumor suppressor genes and of possible negative growth regulators came from the identification of genes involved in the study of familiar cancer. The essential features was that the affected individual inherits a mutant 'loss of function' allele from the affected parent and then a somatic event inactivates the normal allele inherited from the other parent. In contrast, the sporadic forms of the tumor involve two somatic mutational events the second of which must occur in the descendants of the cells that received the first mutation. Thus many tumor suppressor genes have been identified on the basis of a tight

association between neoplasia and the loss of function in both copies of the gene. The existence of 13 tumor suppressor genes was predicted on the basis of such an association but molecular clones are available only for five of them: Rb, p53, WT1, DCC and NF1. These genes are differently localized within the cell: from the extracellular to the nuclear compartments.

However another category of growth inhibitory genes, when absent, might cause death during embryogenesis. The mammalian prohibitin gene, analogue of the developmental Cc gene in Drosophila, was the first member of this hypothetical class to be cloned (99) (117).

A family of RAS-related transformation suppressor genes, the KREV/RAP genes, has also been demontrated to be directly antiproliferative. Moreover the gene that maps at the BTG1 locus involved in t(8;12) (q24;q22) chromosomal translocations in B cell chronic leukemia has been recently shown to accumulate in contact inhibited fibroblasts and to be able to regulate negatively cell proliferation in NIH3T3 cells (139).

3.6.1 DCC: the role of the extracellular matrix.

The evolution of the colonrectal carcinoma from a polyp stage involves, in the majority of cases, the inactivation of a gene localized on chromosome 18 named DCC (Deleted in Colon rectal Carcinoma) (107) (172). This gene was cloned through a physical mapping approach using polimorphic DNA markers that show a loss of heterozygosity on 18q21.3. This gene encodes for a 190 Kd transmembrane phosphoprotein that presents some extracellular domain highly similar to fibronectin type III and C2

macroglobulin-like regions typically present in cell adhesion molecules.

This finding suggests that the extracellular environment has indeed an active role in the restriction of proliferation in a multicellular organisms.

3.6.2 The Neurofibromatosis gene product: interfacing with the ras pathway.

NF1 involves cells of the embryonic neural crest and maps in the 17q11.2 chromosomal region (107) (172). The NF-1 protein has high homology with three proteins that interact with the products of the ras proto-oncogene: the IRA1 and IRA2 from S. cerevisiae and GTPase-activating protein (GAP) of mammals. Infact it has been shown that the GAP-related region of NF1 can stimulate GTP hydrolysis on $p21^{ras}$ and substitute for IRA1 in yeast. Both GAP and NF1 appear to interact with $p21^{ras}$ through the domain that is genetically defined as the site of interaction of $p21^{ras}$ with its target. Therefore it could be that NF1 protein is an effector of $p21^{ras}$. On the contrary NF1 could represent the negative regulator of $p21^{ras}$ since inhibition of its activity would allow the level of $p21^{ras}$:GTP to increase and provide an oncogenically activated signal.

3.6.3 The Wilms' Tumor gene product: interfacing with the 'immediate early' response genes.

The locus involved in the Wilms' Tumor of the kidney map at the human chromosome 11p13 (107) (172). The WT-1 gene codes

for a zinc-finger containing a protein that shares sequence similarities with a mammalian 'immediate early' protein variously termed EGR-1, TIS-8, KNOX-24, NGF1-A and Zif268 (104). Interestingly EGR-1 and WT-1 bind strongly to the same DNA sequence and act as functional antagonists: WT-1 is a transcriptional suppressor while EGR-1 is an activator. Thus the expression of WT-1 in certain embryonal kidney precursor may interrupt the growth program in these cells by antagonizing the ubiquitously expressed EGR-1 that is allowing these cells to reach a final differentiation stage.

3.6.4 The Retinoblastoma gene product: interfacing with cell cycle machinery.

The Rb gene maps to 13q14 and encodes a 105 Kd nuclear phosphoprotein. Its tissue distribution would imply partecipation in growth regulation in a variety of cell and tumor types. However Rb gene inactivation seems to be restricted to a narrow subset of tumors. The phosphorylation state appears to be tightly regulated during the cell cycle. It is infact hypophosphorylated in quiescent cells and during G1, but becomes hyperphosphorylated in late G1/S and G2 (69). In cycling cells Rb reverts to an underphosphorylated state at M/G1 transition. Microinjection of Rb protein that lacks the phosphorylation target aminoacids is able to inhibit G1 progression in SAOS-2 osteosarcoma cells that lack an Rb functional gene. These experiments demonstrate that Rb is truly involved in G1 progression control and that its growth inhibiting property is structurally linked to its dephosphorylated

form. Moreover cells transfected with Rb cDNA's show reduced transcription of c-fos.

This protein exists within DNA tumor virus-transformed cells in the form of a complex with various virus-encoded oncoproteins. Human adenovirus, SV40 and human papillomavirus (HPV) contain an oncoprotein, E1A, LT antigen and E7, that is able to interact with the host cellular pRb. This property suggests that DNA tumor viruses transform cells through their ability to inactivate negative cell growth regulation.

The various viral oncoproteins bind exclusively to the hypophosphorylated Rb leading to the conclusion that the hypophosphorylated form is responsible for Rb mediated growth suppression. The viral oncoproteins share a small region of structural similarity that appears to be involved in mediating complex formation with pRB. Most intriguing is the observation that this oncoprotein-binding region is almost invariably affected in mutant pRb protein isolated from human tumor samples.

Moreover it seems that these viral oncoproteins probably can mimic a cellular protein that must bind to pRb in order to exert its growth suppressing action. Infact recombinant forms of pRb binding domain have been used to demonstrate specific associations with a number of host cell proteins. Kaelin et al. demonstrated the existence of at least eight cellular binding partners for pRb using a fusion protein including the LT-binding domain. It was demonstrated that a Rb-containing protein complex is capable of sequence specific DNA binding. Analysis of the DNA sequences that are selectively bound by the complex demontrated that the majority were similar to the E2F recognition sequence TTTCGCGC which implies that one of the

cellular proteins complexed to Rb was E2F or a E2F-like protein. E2F is a cellular transcription factor that is involved in the E1A activation of a number of cellular or viral genes. A single E2F site can confer E1A regulation to a test promoter and E2F DNA binding activity is elevated after adenovirus infection. Although E2F specifically recognizes and binds to sites within the E2 promoter, this interaction is unstable. The stability is enhanced in the presence of the viral E4 gene that forms complex with DNA binding activity and capacity to activate transcription. Subsequent experiments demonstrated that E2F was found in a heterodimeric complex with other cellular proteins which prevented its interaction with E4. It was found that E1A protein could disrupt those heterodimeric complexes releasing E2F to interact with E4 and that this ability was fundamental for its transforming ability. Thus an initial E2F containing complex was disrupted by E1A, which results in the release of free E2F. Several observations suggest that the interaction of Rb with E2F is absolutely significant with respect to the action of Rb as a tumor suppressor. No E2F-Rb complex is found in cells that produce mutated Rb that is the E2F-Rb complex is disrupted by each viral oncoprotein previously shown to bind to Rb (115).

However extracts from synchronized NIH3T3 cells contain at least two other E2F-containing complexes. One complex accumulates during the G1 phase of the cell cycle and then disappears at the end of G1. A second distinct complex appears at the beginning of the S phase, accumulates during the S phase and then disappears. Both the complexes contain the pRb-related p107 protein. This protein has been shown previously to be bound to E1A in adenovirus-infected cells. p107 and E2F seem to be

directly associated and together change their partners during G1 progression. In G1 they are able to bind the complex Cyclin E-cdk2 while in S phase they bind the complex cyclin A-cdk2. Co-transfection of pRb and p107 expression plasmid are able to inhibit E2F-dependent transcription suggesting that the transcriptionally active form of E2F may be the form that is free of interactions with cellular proteins such pRb or p107. c-myc and cdc2 genes have been shown to be repressed by Rb in a E2F dependent manner. In this context, the complex Rb-E2F would not simply inhibit the transcriptional function of E2F but would create a dominant repressor complex. The complex p107-E2F-$p33^{cdk2}$ is a cyclin dependent complex that is involved in G1 progression. The complex with Cyclin A is specific for S phase and is able to specifically binds to E2F site DNA and has H1 kinase activity. It is possible that it activates E2F-dependent promoter at the start of the S phase by phosphorylating components of the transcriptional apparatus bound to adjacent segments of the promoter. From this point of view, p107 is a growth promoting factor. Many genes have E2F binding sites in their own promoter. These genes are mainly activated in late G1 and code for proteins required for DNA synthesis.

Moreover constitutively expressed cyclin A and E can overcome pRb mediated suppression of proliferation. pRb becomes hyperphosphorylated in cell overexpressing these cyclins and this phosphorylation is associated for cyclin A and cyclin E mediated rescue of pRb blocked cells (76).

3.6.5 p53: checkpoints in mammalian cells.

Loss of heterozygosity of markers on the short arm of chromosome 17 occurs at high frequency in several tumors and this mutation resides on the p53 gene. wt p53 is a growth suppressor and an inhibitor of transformation (94) (97) (168) (182).

The activation of p53 appears to be controlled by regulation of p53 levels and by phosphorylation: its level is very low after mitosis but increases in G1 and during S phase becomes phosphorylated. Both cdc2 and casein kinase II phosphorylate p53.

p53 binds DNA specifically and contains an acidic activation domain at its N-terminus. Moreover cotransfection of a p53 expression vector with a plasmid containing a p53-binding site upstream of a reporter gene results in a high level of reporter activation in mammalian cells. On the contrary mutants from the 4 hot region lose the ability to bind p53-binding site and accordingly cannot activate the expression of adjacent reporter genes.

In addition to intragenic mutations of p53, alterations of other genes can lead to the same physiological consequence. The first example was provided by DNA tumor viral oncoproteins. LT antigen and E1B are able to form complexes with p53 that increase p53 half-life but inhibit the enter into the nucleus. The Papilloma virus protein E6 seems to achieve a similar end result but in a different manner: by associating with recently synthesized p53 molecule it brings them to rapid destruction apparently using the ubiquitin-dependent proteolitic machinery.

The p53 pathway may also be disrupted by activation of a cellular gene, MDM2, a gene amplified in a significant fraction of human sarcomas and that has recently been shown to bind to p53. Moreover p53 can block the progression of the cell cycle when artificially expressed at high levels. Emerging evidence, however, suggests that p53 may play an important growth controlling role in stressed cells. In response to X ray or drug induced DNA damage, normal cells increase p53 expression and are arrested in the cell cycle until the damage is repaired. In contrast, cells with mutant p53 genes are only partially blocked, continue to divide and die. Recently it has been shown that mutations in p53 were sufficient to allow gene amplification to occur. Moreover expression of wt p53 in tumor cells containing mutated p53 restores G1 control and reduced the frequency of gene amplification to undetectable levels. These observations reveal that p53 contributes to a metabolically regulated G1 checkpoint that controls the conditions of genomic DNA before replication (113) (71).

RESULTS

1. Cloning and characterization of *sdr*.

1.1 Introduction.

In the past years it has been a long debate on the mechanisms that relate the growth arrest mediated by serum starvation to that induced by contact inhibition. The phenomenon of contact inhibition was first described as cessation of cell movement when two adjacent cells come into contact; by extension this concept was then applied to the consequent inhibition of cell division (2) (47) (78) (158) (165).

Since then a debate focussed on the relevance of cell to cell contact for the establishment of the quiescent state. Theoretically the arrest of cells in an overcrowded culture could be due to depletion of the available growth factors in the medium thus totally resembling the arrest caused by deprivation of mitogens. Accordingly, even the increased growth at the margin of a wound in a confluent monolayer was explained as a local increase of growth factor concentration.

All the data accumulated so far have not been able to dissect the tight relationship between the two growth arrest states. However recently a specific increase of a tyrosine phosphatase activity has been reported in density dependent growth arrested but not in growing or serum deprived Swiss 3T3 cells (120).

I thus decided to investigate the existence of potential differences in gene expression between the two quiescent states.

Moreover the characteristic pattern of expression of the gene isolated during the course of this study has allowed me to dissect the control elicited on gene expression by different growth factors during G1 progression network of signal transduction pathways.

1.2 Cloning of genes highly induced in serum deprived cells but not in serum stimulated and density dependent growth inhibited cells.

An oriented cDNA library was prepared from the poly(A)+ RNA fraction isolated from NIH3T3 cells starved for 48 hours in 0.5% FCS. Under these conditions less than 5% of cells were in S Phase.

In order to clone genes that are specifically related to the growth arrest state induced by serum deprivation, I decided to screen the Go serum starved cDNA library with three cDNA probes synthetized from: a. resting NIH3T3 cells cultured for 48 hours in low serum; b. serum stimulated cells at 6 hours after addition of 20% FCS to starved cells; c. density dependent growth inhibited cells that have been cultured for 8 days with the medium replaced every 2 days.

Using this approach I was able to isolate genes that are highly expressed in serum starved cells and down-regulated during Go->G1 transition and most importantly to discriminate the ones that are not induced in density dependent growth inhibition. Clones that are highly expressed in serum starved but

not in serum stimulated and density dependent growth arrested cells were purified, rescreened with the respective cDNAs probes and further analyzed.

After restriction and cross-hybridization analysis, 35 plaques appeared to be cDNAs of different lengths of the same mRNA species.

The longest clone was 3 Kb. It was named *sdr* (serum deprivation response) and further characterized.

1.3 Responsiveness of *sdr* mRNA after serum starvation and density dependent growth inhibition.

The level of *sdr* mRNA expression was analyzed on Northern blots by using total RNA extracted at various times after serum deprivation of exponentially growing NIH3T3. Cells were shifted both to 0.5% FCS or to serum free medium 24 hours after seeding in 10% FCS. *sdr* probe recognizes a 3 Kb long transcript that is normally expressed in growing cells and then increases during the initial 6 hours of 0.5% serum addition. Later it is transiently down-regulated at 24 hours and again highly induced after 48 hours (Fig. 1; panel A). Surprisingly the addition of serum free medium to growing cells down-regulates *sdr* mRNA expression within 6 hours; later it is again up-regulated at 24 hours and reaches its maximum level after 48 hours (Fig. 1; panel B).

Normalization of RNA amounts was performed by probing the same Northern blot with *gapdh*.

The percentage of cells in S phase was monitored by analysing BrdU incorporation in cells grown on cover slips for

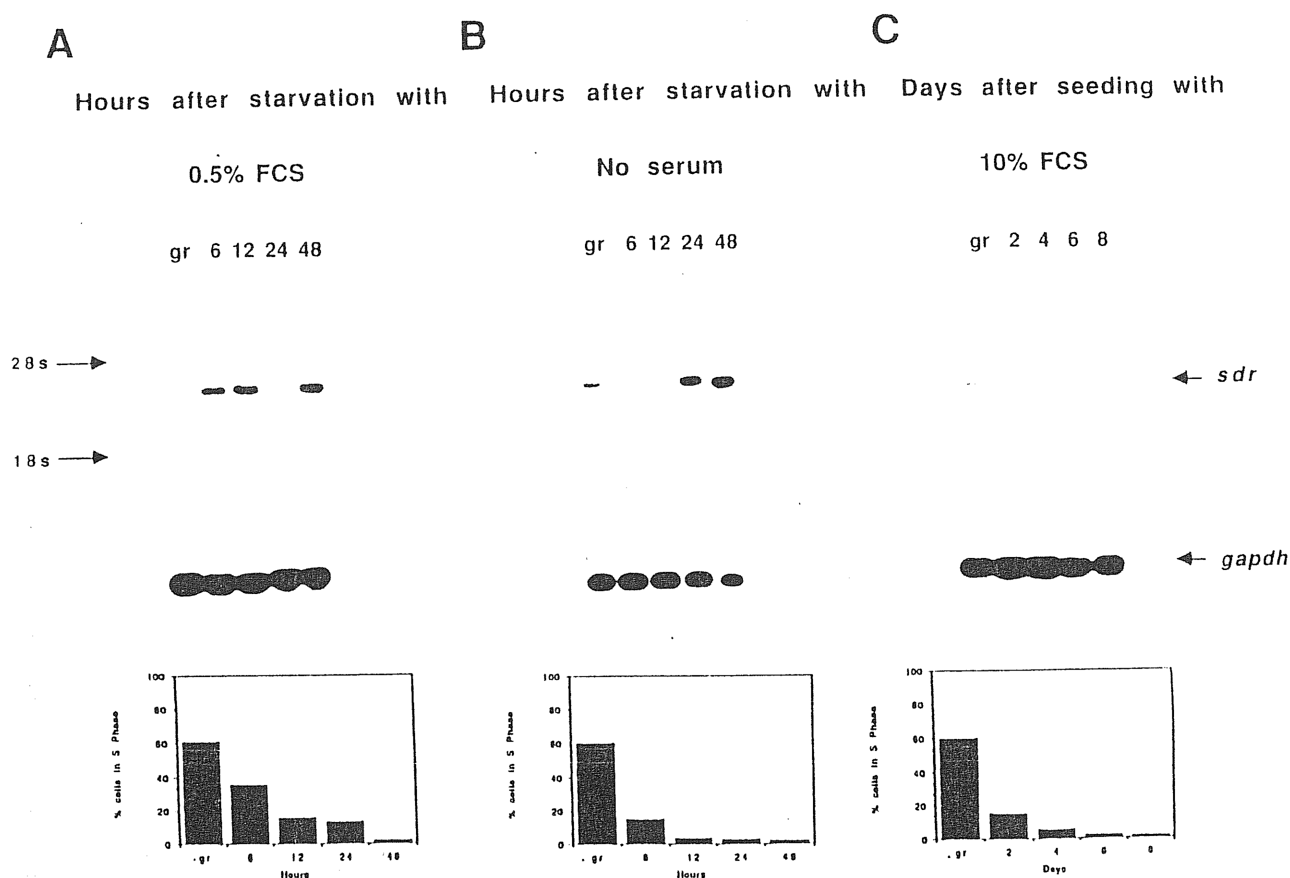

Fig. 1. Analysis of *sdr* mRNA level during growth arrest induction.

Northern blot analysis was performed on equal amounts of total RNA (10 μg) isolated from panel A) growing NIH3T3 cells and at the indicated times after 0.5% FCS addition panel B) growing cells and at the indicated times after serum free medium addition panel C) actively growing cells that were kept thereafter in the same dish with 10% FCS for different times (culture medium containing 10% FCS was replaced every two days). The same blot was probed, as indicated, with *sdr* and *gapdh* cDNAs. The analysis of DNA synthesis levels for each time course is shown at the bottom.

each time point. Less than 15% of the cells remained in S phase 24 hours after serum starvation.

To assess the expression of *sdr* mRNA during density dependent growth inhibition, NIH3T3 cells were seeded in 10% FCS, and every 2 days the medium was replaced with fresh 10% FCS. The *sdr* mRNA level in growing cells is down-regulated as soon as 2 days after seeding and is maintained at such level during the complete time course (Fig. 1; panel C).

gapdh control expression level did not change, and DNA synthesis analysis showed a significant decrease as early as 2 days after seeding.

1.4 Regulation of *sdr* mRNA during Go->S transition.

Fig. 2 shows the expression of *sdr* mRNA at different times after a synchronous induction of the cell cycle with 20% FCS in NIH3T3 cells that have been previously arrested for 48 hours in 0.5% FCS. The mRNA identified by *sdr* probe is abundantly expressed in arrested cells (time 0) and has the lowest expression 6 hours after addition of serum. Surprisingly the mRNA increases after 12 hours from serum addition and becomes down-regulated again at 18 hours.

The same filter was hybridized with the *gapdh* cDNA probe to normalize each RNA sample loaded on the Northern blot. The percentage of cells in S phase is also shown.

Hours after addition of

20% FCS

0 3 6 9 12 18 22

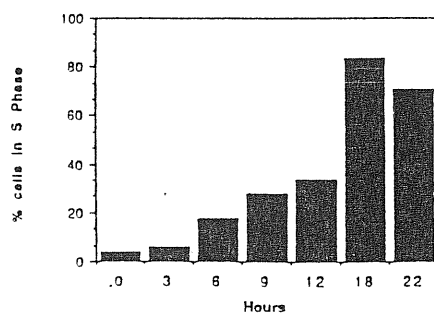

Fig. 2. Kinetics of *sdr* mRNA expression during Go->G1 transition.

NIH3T3 cells were arrested for 48 hours in 0.5% FCS and stimulated to reenter the cell cycle with the addition of 20% FCS. After the indicated intervals, RNA was isolated and analyzed by Northern blot (10 µg of total RNA) with *sdr* and *gapdh* probes. The percentage of cells in S phase is shown at the bottom of each time course.

1.5 Response of *sdr* to single growth factor addition.

In order to better understand *sdr* gene regulation, I decided to use single factors to elicit a growth response in NIH3T3 cells.

Fig. 3; panel A shows *sdr* expression after addition of PDGF: its mRNA is down-regulated after 6 hours from growth factor addition and then becomes highly expressed 12 hours after. The lowest level of expression is reached 18 hours from PDGF addition; *sdr* mRNA is again reinduced at 22 and 30 hours.

Similarly to PDGF, Insulin is able to down-regulate strongly *sdr* mRNA expression after 6 hours (Fig. 3; panel B). This effect is followed by an initial increase in the expression of the gene and a subsequent new strong inhibition. After 30 hours from the addition of Insulin *sdr* is again highly expressed.

FGF is able to down-regulate the expression of *sdr* after 6 hours. At 12 hours the level of expression increases but never reaches the amount of quiescence (Fig. 4; panel A).

TPA has an effect on *sdr* mRNA expression similar to FGF (Fig. 4; panel B).

Like the other mitogenic factors tested, EGF is able to completely prevent *sdr* mRNA expression within the first 6 hours (Fig. 5). Surprisingly this growth factor is the only one that is able to completely inhibit the reinduction of *sdr* mRNA in the G1->S transition.

Normalization of RNA amounts was performed with the *gapdh* probe on the same blot. The percentage of cells in S phase

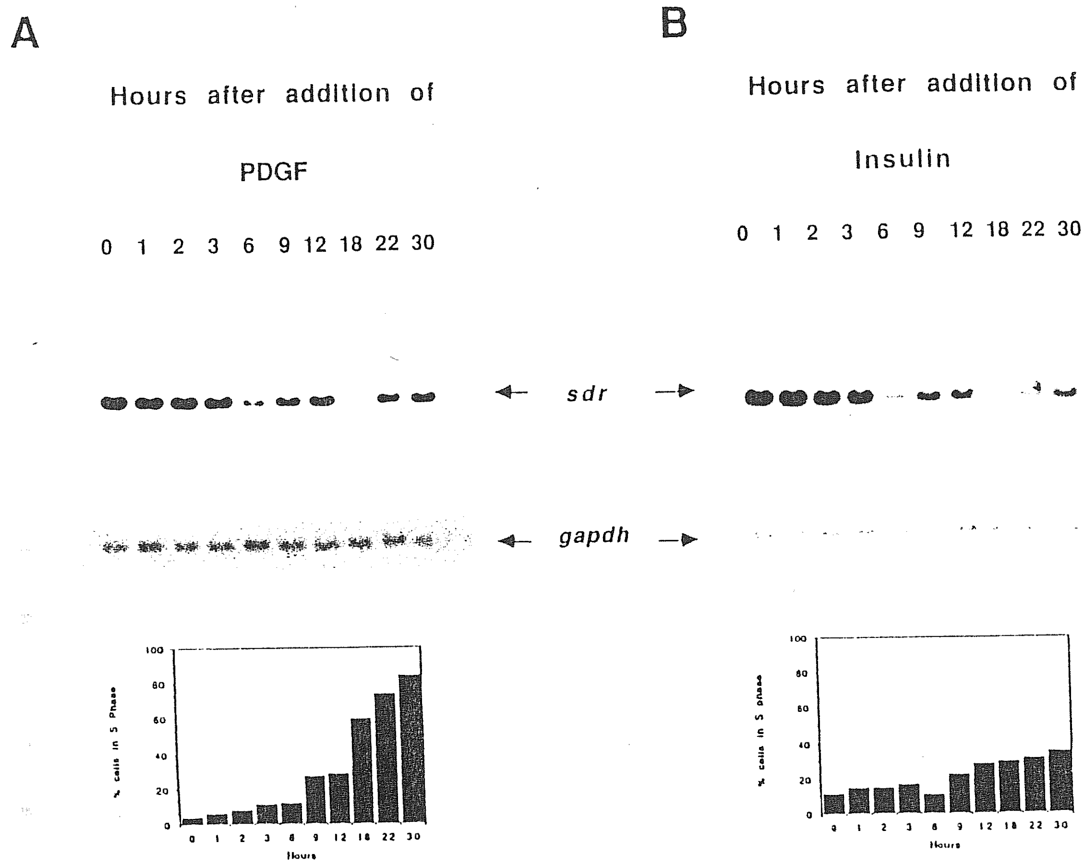

Fig. 3. Regulation of *sdr* mRNA by PDGF and Insulin.

NIH3T3 cells were made quiescent by 48 hours in 0.5% FCS and stimulated with the addition of serum free medium containing 20 ng/ml of PDGF (panel A) or 100 µg/ml of Insulin (panel B). RNA was extracted at the described times and analyzed by Northern blots (10 µg) with *sdr* and *gapdh* probes. Analysis of cells in S phase is also shown.

was measured by analizing BrdU incorporation in cells growing on cover slips for each time point for every growth factor and TPA.

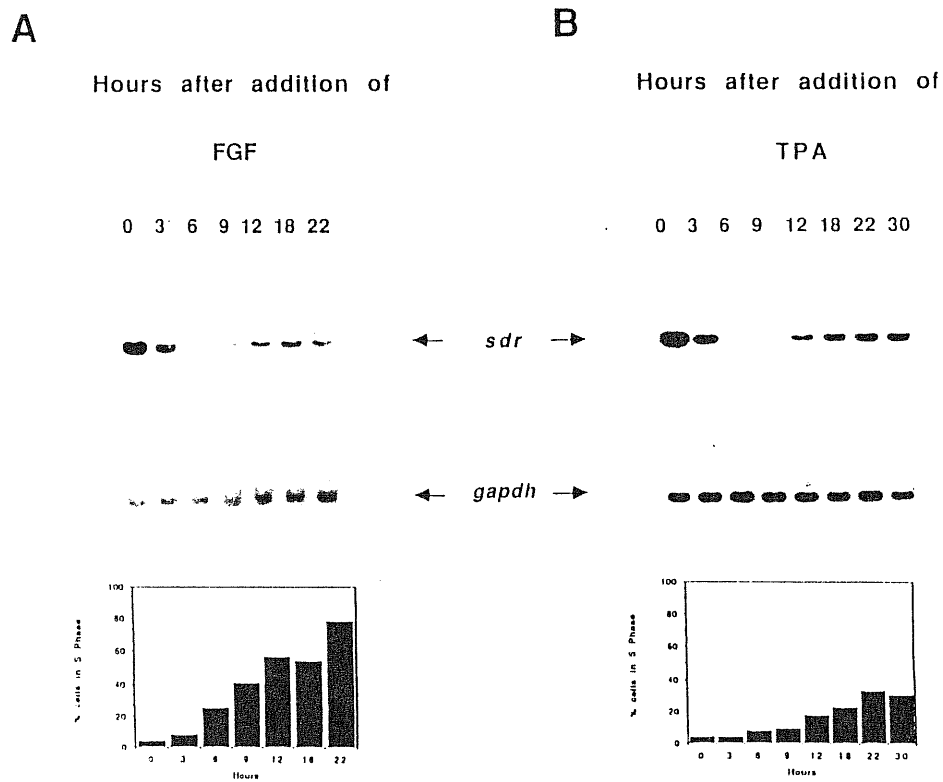

Fig. 4. Regulation of *sdr* mRNA by FGF and TPA.

NIH3T3 cells were made quiescent by 48 hours in 0.5% FCS and stimulated with the addition of serum free medium containing 100 ng/ml of FGF (panel A) or 10^{-7} M TPA (panel B). RNA was extracted at the indicated times and analyzed by Northern blots (10 µg) with *sdr* and *gapdh* probes. Analysis of DNA synthesis is also shown at the bottom.

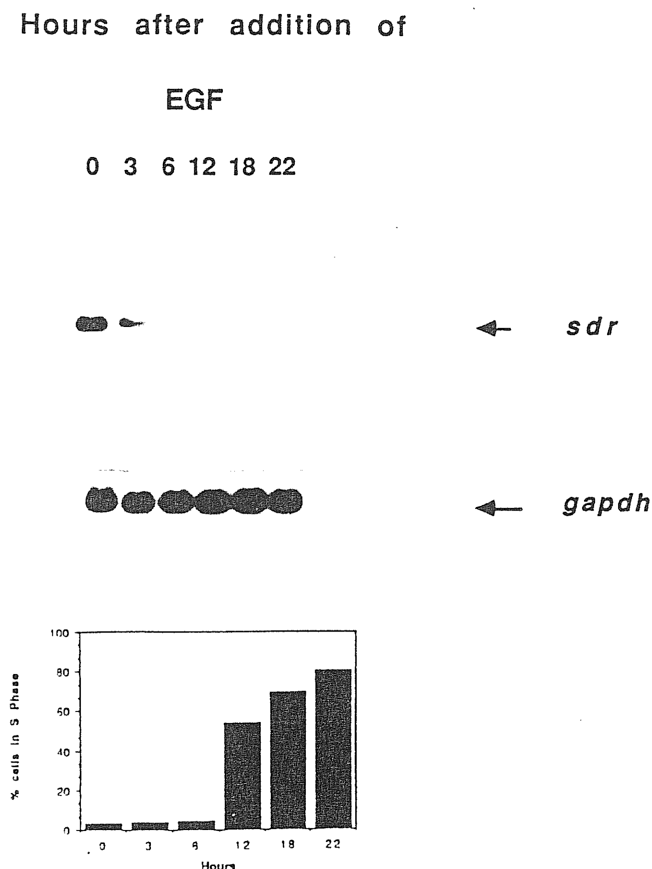

Fig. 5. Regulation of *sdr* mRNA by EGF.

NIH3T3 cells were arrested for 48 hours in 0.5% FCS and stimulated to reenter the cell cycle with the addition of serum free medium containing 40 ng/ml of EGF. After the indicated intervals, RNA was isolated and analyzed by Northern blot (10 µg of total RNA) with *sdr* and *gapdh* probes. The percentage of cells in S phase is shown at the bottom of each time course.

1.6 Postranscriptional regulation of *sdr* mRNA expression.

I studied and compared the molecular mechanisms involved in *sdr* control in different growth states: quiescence, Go->G1 and G1->S transitions in serum and EGF stimulated cells.

First I performed a nuclear run-on experiment, shown in Fig. 6, to assess whether transcriptional regulation is responsible for the decreased expression of *sdr* mRNA after serum addition to arrested NIH3T3 cells. Nuclei collected at various times after FCS addition to Go NIH3T3 cells synthesize *sdr* mRNA at the same level before and after growth induction. As controls, the *gapdh* transcriptional level remains constant while the transcription of *gas1* mRNA is strongly down-regulated 6 hours from the addition of serum (Fig. 6) (23). From this result I can conclude that the expression of *sdr* mRNA is mainly regulated at the posttranscriptional level.

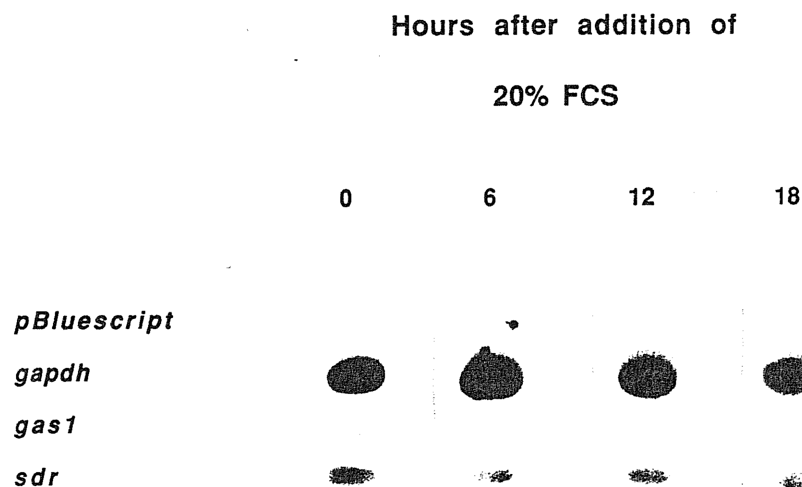

Fig. 6. Nuclear run-on analysis of *sdr* transcription during the growth cycle.

Nuclei were isolated from NIH3T3 cells at growth arrest (48 hours in 0.5% FCS) and at various times after 20% FCS addition. The nuclear preparations were allowed to incorporate [α-^{32}P] UTP and the recovered [^{32}P] labelled RNA was allowed to hybridize to denatured and immobilized plasmid DNA containing the indicated cDNA inserts.

I thus analyzed *sdr* mRNA stability in serum deprived Go NIH3T3 cells either alone or in the presence of Actinomycin D or Cycloheximide to block further mRNA transcription or protein synthesis respectively.

Fig. 7; panel A shows that *sdr* mRNA half-life is relatively long (at least 4 hours) and is not affected under growth arrest conditions either by Actinomycin D or Cycloheximide.

Next I analyzed the mechanism of serum (20% FCS) or EGF induced *sdr* mRNA instability. Fig. 7; panel B shows that both Actinomycin D and Cycloheximide inhibit the early serum induced down-regulation of *sdr* mRNA, thus pointing to a clear requirement for both RNA and protein synthesis in the serum response of *sdr* mRNA. In the case of EGF, the Actinomycin D effect on *sdr* expression is strikingly decreased suggesting the existence of a transcription- independent mechanism which could also be responsible for maintaining *sdr* mRNA down-regulation at later times.

The *sdr* expression during G1->S transition was similarly dissected as can be seen in Fig. 7; panel C. While Actinomycin D is able to shut off the serum dependent *sdr* mRNA reinduction pointing to a requirement for active transcription, Cycloheximide is able to superinduce its expression during serum stimulation and unexpectedly also during EGF treatment. CHX effect thus seems to reflect an active process of down-regulation which is dependent on protein biosynthesis.

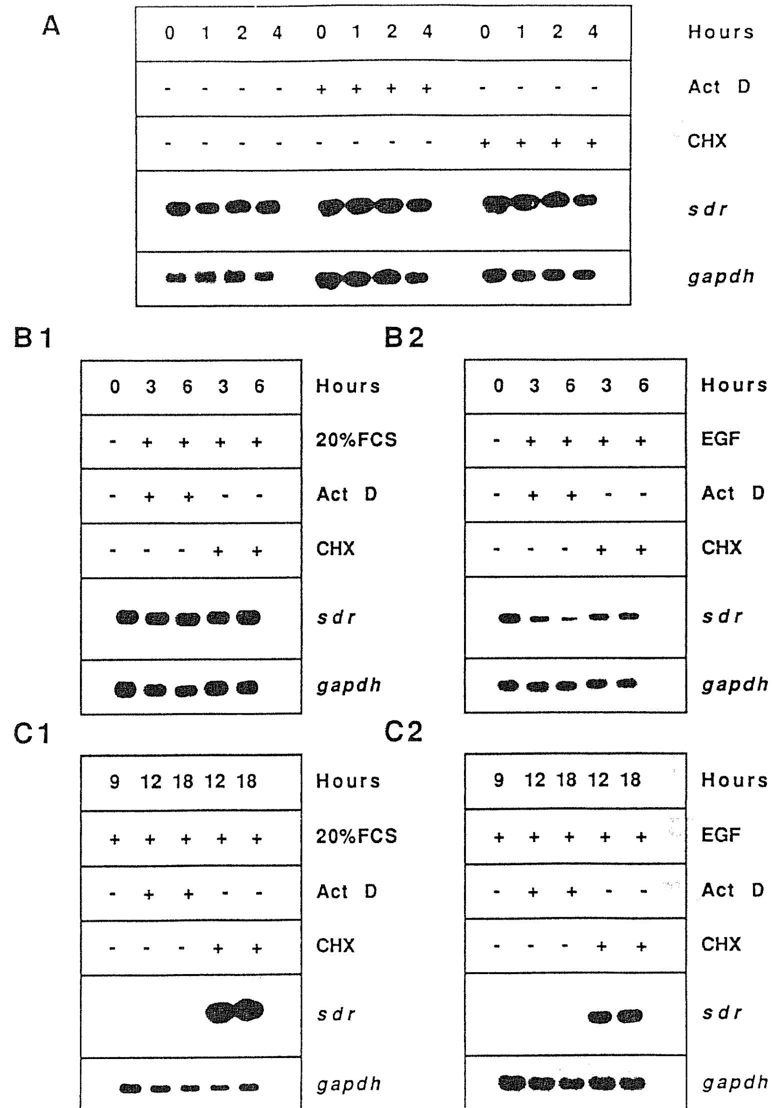

Fig. 7. Effects of Actinomycin D and Cycloheximide on *sdr* mRNA.

sdr and *gapdh* cDNAs were used as probes in Norhern blot analysis of total RNA (10 µg) extracted from: panel A) 48 hours in 0.5% FCS and at the indicated times in the absence (-) or presence (+) of Actinomicin D or Cycloheximide; 20% FCS (panel B1) or EGF (panel B2) activated cells in the presence of Actinomicin D or Cycloheximide. In panel C the two drugs were added after 9 hours from the addition of 20% FCS (C1) or EGF (C2).

1.7 Expression of *sdr* mRNA in different tissues.

In order to analyze the *sdr* mRNA level in vivo, total RNA was extracted from different mouse tissues. By Northern blot analysis, *sdr* results highly expressed in lung; it is also highly expressed in heart and kidney. The other analyzed tissues (liver, spleen, stomach and uterus) present a detectable level of *sdr* expression. The size of *sdr* mRNA is apparently similar in all the tissues analyzed as observed in NIH3T3 cells (Fig. 8).

Fig. 8. Expression of *sdr* mRNA in mouse tissues.

Total RNA was extracted from murine organs and analyzed on Northern blot (20 μg) using *sdr* cDNA as probe. Tissues indicated are as follows: Lu=lung; He=heart; Ut=uterus; Sp=spleen; Ki=kidney; St=stomach; Li=liver; 3T3= NIH3T3 cells for 48 hours in 0.5% FCS.

1.8 *sdr* cDNA sequence.

The complete sequence of the longest *sdr* cDNA clone comprises 2909 nucleotides (Table 1). The 5'UTR is 118 nucleotides long and contains stop codons in all three ORFs. A large open reading frame of 1254 nucleotides encodes for a protein of 418 aminoacids with a predicted molecular weight of 46.6 Kd. The ATG at position 119 resembles the Kozak's consensus sequences for the initiation of translation (87). The 3'UTR, showing frequent stop codons in all three frames, is 1537 nucleotides long. It contains two consensus elements (AUUUA) involved in mRNA stability (148) and two polyadenylation signals: one, AGUAAA, is rarely found in Eukaryotic mRNAs and is positioned far from the 3'end of the mRNA at -477 (149), the other, AUUAAA, is the most common variant and is at -32 nucleotides.

Table 1.

DNA and deduced protein sequence of the cDNA clone of *sdr* gene. The 3' untranslated sequence shows two putative instability motifs ATTTA (underlined) and two consensus signals AGTAAA and ATTAAA for poly(A) addition at position 2432 and 2878.

```
   1 AAGCCAAAACCTCTGCTAGCCCTAGAGAGCAGTGCTCACTTCAGACCAACCAGCCTCCTG
  61 TGCTTCTAGCTTGAGCAAGAAGTTTCCAGCGGGGAAGTAAGTTTCTAGCTGCCCAGCCAT
                                                             M   1

 121 GGGAGAGGACGCTGCACAGGCAGAAAAGTTCCAGCATCCAAACACAGACATGCTCCAGGA
      G  E  D  A  A  Q  A  E  K  F  Q  H  P  N  T  D  M  L  Q  E  21

 181 GAAGCCATCCAGCCCCAGCCCAATGCCTTCCTCCACACCGAGCCCCAGCCTGAACCTGGG
      K  P  S  S  P  S  P  M  P  S  S  T  P  S  P  S  L  N  L  G  41

 241 GTCCACAGAGGAGGCCATCCGAGACAACTCGCAGGTGAATGCTGTCACCGTGCACACACT
      S  T  E  E  A  I  R  D  N  S  Q  V  N  A  V  T  V  H  T  L  61

 301 CCTGGATAAATTGGTCAACATGCTGGACGCCGTGAGGGAGAACCAGCACAACATGGAACA
      L  D  K  L  V  N  M  L  D  A  V  R  E  N  Q  H  N  M  E  Q  81

 361 GCGTCAGATCAACCTGGAGGGCTCGGTGAAGGGCATCCAGAACGACCTCACCAAGCTCTC
      R  Q  I  N  L  E  G  S  V  K  G  I  Q  N  D  L  T  K  L  S  101

 421 CAAGTACCAGGCCTCCACCAGCAACACAGTGAGCAAGCTGCTAGAGAAGTCTCGCAAGGT
      K  Y  Q  A  S  T  S  N  T  V  S  K  L  L  E  K  S  R  K  V  121

 481 CAGCGCTCACACGCGGGCTGTCCGGGAGCGCCTCGAGAGGCAGTGTGTCCAGGTGAAGAG
      S  A  H  T  R  A  V  R  E  R  L  E  R  Q  C  V  Q  V  K  R  141

 541 ACTGGAGAACAACCACGCCCAACTCCTCCGACGCAACCACTTCAAAGTGCTCATCTTCCA
      L  E  N  N  H  A  Q  L  L  R  R  N  H  F  K  V  L  I  F  Q  161

 601 GGAAGAAAGTGAGATCCCTGCCAGTGTGTTTGTGAAGGAGCCAGTTCCCAGCGCTGCAGA
      E  E  S  E  I  P  A  S  V  F  V  K  E  P  V  P  S  A  A  E  181

 661 AGGCAAGGAGGAGCTTGCTGATGAGAACAAGTCCCTGGAGGAAACTCTGCACAACGTGGA
      G  K  E  E  L  A  D  E  N  K  S  L  E  E  T  L  H  N  V  D  201

 721 CCTCTCCTCTGATGACGAATTGCCCCGTGATGAGGAAGCCCTGGAAGATAGTGCAGAAGA
      L  S  S  D  D  E  L  P  R  D  E  E  A  L  E  D  S  A  E  E  221

 781 GAAGATGGAAGAAAGCAGGGCAGAAAAAATAAAAAGATCCAGCCTCAAGAAAGTCGATAG
      K  M  E  E  S  R  A  E  K  I  K  R  S  S  L  K  K  V  D  S  241

 841 CCTCAAGAAAGCGTTTTCTCGTCAGAACATCGAGAAAAAGATGAACAAGCTGGGAACCAA
      L  K  K  A  F  S  R  Q  N  I  E  K  K  M  N  K  L  G  T  K  261

 901 GATCGTATCTGTTGAGAGGAGAGAAAAGATTAAGAAATCGCTCACACCCAACCACCAGAA
      I  V  S  V  E  R  R  E  K  I  K  K  S  L  T  P  N  H  Q  K  281

 961 AGCATCTTCTGGGAAAAGCTCCCCCTTCAAGGTTTCTCCCCTCTCCTTTGGTCGTAAGAA
      A  S  S  G  K  S  S  P  F  K  V  S  P  L  S  F  G  R  K  K  301

1021 AGTCCGAGAGGGAGAAAGCTCTGTAGAAAATGAGACCAAGTTGGAAGACCAGATGCAGGA
      V  R  E  G  E  S  S  V  E  N  E  T  K  L  E  D  Q  M  Q  E  321

1081 GGACCGCGAGGAGGGTTCGTTCACAGAGGGTCTTTCTGAAGCATCCCTCCCCAGTGGCCT
      D  R  E  E  G  S  F  T  E  G  L  S  E  A  S  L  P  S  G  L  341

1141 GATGGAGGGCAGCGCAGAAGATGCTGAGAAATCAGCAAGGAGAGGGAACAACTCAGCCGT
      M  E  G  S  A  E  D  A  E  K  S  A  R  R  G  N  N  S  A  V  361

1201 GGGCAGTAATGCGGATCTGACCATTGAGGAAGATGAAGAAGAGGAGCCGGTAGCCCTGCA
      G  S  N  A  D  L  T  I  E  E  D  E  E  E  E  P  V  A  L  Q  381

1261 GCAGGCCCAGCAGGTGCGCTATGAGAGTGGCTACATGCTCAACTCCGAGGAGATGGAGGA
      Q  A  Q  Q  V  R  Y  E  S  G  Y  M  L  N  S  E  E  M  E  E  401

1321 ACCCAGTGAGAAACAGGTCCAGCCAGCTGTGCTGCATGTGGATCAGACTGCCTGAGTGCA
      P  S  E  K  Q  V  Q  P  A  V  L  H  V  D  Q  T  A           418

1381 CAGCCAGAACACTGGGCCAGTGCATCCACCTCGTGGAAGCCACCCCGCACCTCTTCATCT
1441 CTACCTGCTCCCTACTGTGACTGTCCAGGCCTTAATCACTGACCGTACAGTAGCCTCAAT
1501 GGGATCACAGAAACAAACTGCCAGGAATCCCATTTCTAATTTGCTCATAGGTTCTATTTC
1561 TGTAGAGTTTCTCCAAGATTGCAAAAAGAAAGAAATGGAGCAGTTAAAAAAAAAAAATCAA
1621 CTATCTCTTTGGCTTAGTCAGAAAAACTGGAGGATATTTAAGTTAGTAGTTATAAAAAGT
1681 GATTTTTTTTTTGGTCATTCTATCAGCTTGCTGAATAGTGTAGCAGTAATGTAGTGAATT
1741 CTTACTGTATTCTGTGAATGATTATTCTTCAAGTATGTGATATGGAATATACATAAGTCT
1801 GTAAGCTGAGAATATAACTCTTTATTTTAAGAAGAAAACATTTACAAAAGATAAAGCTGA
1861 AGGACCAGCTGGTTATTCTCTTATAGAAACCATTGTTTGTTGTAAGGGGGCTGAAAGACG
1921 GTGCTGATGGTAAGCTAATAGCACCAGGACTGCTGAGATAGAAAAATTAAGATCAAAAAG
1981 GTTGGAAAGTGGGAGAAATGGAGAACAAACAATGCCAAAAATAATGATGACAAAGAGAAA
2041 AAAAAAAGATGTGCTTAAAAAGTGAACTACTAACATTTTAAAACTTATTTTGCTCTTTAA
2101 GAATACTAAAGAAGTTCATATGTCTTGCAAAAATATATCTTATCTTCCCAATATTTTTAT
2161 AGTCTAGTATTATAACCTAACCAGATGTTCTGATGTAAAAACTATGGTTCTTTTCACTAA
2221 ATAAATGGCAGTACACATCTTTTTGTGAAGAAGCTCAAAATAATATTGTCTCTTTAACAT
2281 TGGAACAAGTGTAAGTCTCAAATAAGTAGTATGTACTGTTTTATGGGGATGTCAATCAAA
2341 TGATTATTCAATATTTTTCACTTTGAAGTGACCTTTCCATTCCAAATATGATATAGGTTT
2401 AGGAGGTATTCAAACTTGCATGTTTAAGAACAGTAAAAGCCTGGGTTTTCTTTAAACACA
2461 TAATGGTAATTTCAATCCAGGTAATGTTTTATTTATACATTAATTTCACAGGATATTAAT
2521 GGTTTAGATTATAAAAGCATGCTAGTATTTTATTTCATGTGTACATTTGCTCTAACATAC
2581 TACTTAAAAGTTGCTTTATCTATTTTTCATTACAAAATGCCAATGCTTTGTTATATTGTA
2641 GTCTGAAGATGAGAGACCCAGGGCTCGAACTCTCTACTCTTACTGCGCAGTAGCACAAGT
2701 GATCCTACACACAGTGGTGACTAGGCAGCAGTCCTGGGCTGCACTCTGACTTTCAGAGAA
2761 GGAGTGAGGTGAATAATGGGTGTTGATGGTGTAACCTCTTCAGCTGTGTTCCTGTACTGC
2821 AATTAATGTAACCCCAAATACTGAATTGCTGAACTAACCATGCCTGTGTGAAAGAAGATT
2881 AAATAAACCAAAAATAAAAAGTAAAAAAA
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1.9 *sdr* mapping in the murine genome.

In collaboration with D. M. Colombo (Divisione di Oncologia Sperimentale, Istituto Nazionale Tumori, Milano) the SDR gene was localized in the murine genome. The presence of an RFLP for a restriction enzyme site was scored in a panel of recombinant inbred strains of mice and in the progeny of backcrosses both between laboratory mouse strains and between a laboratory strain and *Mus spretus*. An *sdr* probe containing the 1.8 Kb part at the 5' of the cDNA sequence was able to recognize two bands after an EcoRI digestion of murine genomic DNA: one of them resulted polymorphic. Comparison of the distribution of the RFLP to ones determined previously for other loci indicated linkage of *sdr* to *Aox-1* locus on chromosome 1.

In collaboration with Dr. P. Gros (Department of Biochemistry, McGill University, Montreal) the gene was more finely mapped within this chromosome (Table 2). Dr. Gros' group is interested in the genetic and physical mapping of this part of the murine genome since they have previously shown the localization of the *Bcg* gene in this area. This gene is of interest because it confers infection resistance to a large spectrum of agents like *Mycobacterium lepraemurium* and *Leishmania donovani*. They have previously mapped 40 polimorphic markers to the proximal part of this chromosome in a series of interspecific and intraspecific inbred strains (60 strains) that segregate at *Bcg*. They scored the *sdr* pattern of inheritance in 153 backcrosses. The aplotypes were determined in 139 animals for 10 anchor markers. The gene order and the intergenic distance (in

cM) was the following: *Col3a1*-0.7-*sdr*-5.5-*Leu2*-3.3-*Fn-1*-2.0-*Tp-1*-0.8-*Vil/Des*-0.4-*Inha*-11.5-*Akp-3*-0.8-*Acrg*-2.0-*Sag*.

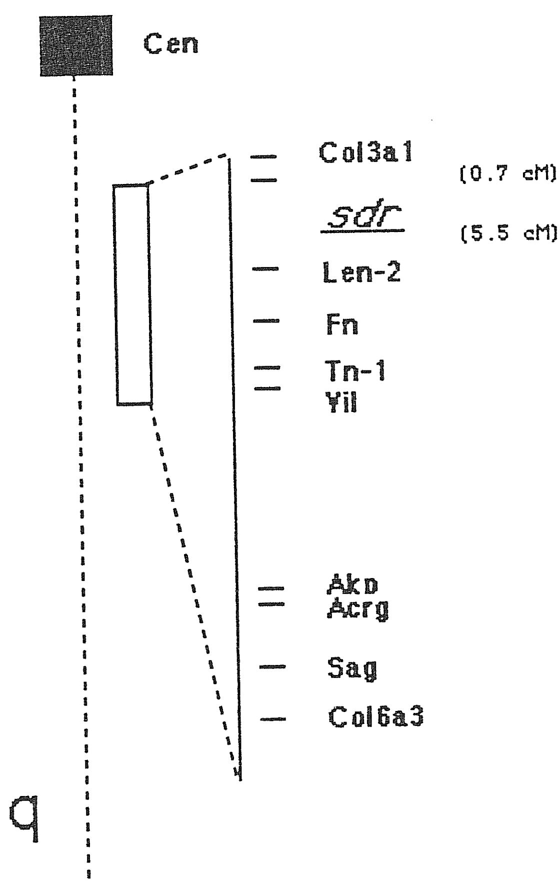


Table 2.

A schematic rapresentation of the linkage map of a portion of murine chromosome 1. The enlarged region is synthenic to a part of the human chromosome 2.

1.10 Isolation of human *sdr* cDNA.

Using the ORF containing region of the murine *sdr* cDNA as probe in a Southern blot analysis under low stringency condition a specific band in human genomic DNAs was detected (data not shown). This result allowed us the cloning of the human homologue of the *sdr* gene. An oriented cDNA library was constructed in λGDST3/T7 vector starting from the poly(A)+ fraction of RNAs purified from normal human liver. 160000 plaques of this library were then screened at low stringency condition (see Materials and Methods) using as probe the murine *sdr* cDNA fragment of 1.7 Kb. One clone was able to cross-hybridize with the probe several times. It was named *hsdr* (human *sdr*).

1.11 *hsdr* cDNA sequence and its homology to the murine *sdr*.

The *hsdr* cDNA fragment isolated was 1779 bp long and completely sequenced. At position 147-149 it contains an ATG codon highly similar to the Kozak consensus sequence for the initiation of translation (87). The 5' untranslated region contains stop codons in all the three frames. The Open Reading Frame ends at nucleotide 1423 and is 1275 nucleotides long. It codes for a deduced protein of 425 aminoacids with a putative molecular weight of 47.2 Kd.

Both the DNA and protein sequences of *hsdr* were compared to the sequences of murine *sdr*. The homology at the nucleotide level is 72% with the highest homology region from nucleotides

148 to nucleotides 1102 (87%) and the lowest one from 1102 to 1425 (68%). These results suggest that the *hsdr* fragment we have isolated contains most of the 5' region of the *sdr* gene and lacks a large portion of the 3' UTR. The deduced *hsdr* protein sequence has 82% of homology with the murine counterpart confirming that *Sdr* protein is highly conserved in Mammals (Table 3; panel A).

1.12 Sequence data bank research of *hsdr* deduced sequence protein.

The predicted *hsdr* aminoacid sequence was compared to the current protein data base release using the Intelligenetics Software Package. Together with interesting similarities with neurofilaments (13%), microtubule associated protein 1B and paramyosin that appear in their α-helical rich domains and that can result from common secondary structures, two more significant homologies were observed:

1. The sequences of three CN-Br-cleavage fragments of the PS-p68 protein resulted to be almost perfectly homologous to the *hsdr* aa 80-109 (peptide A), aa 133-157 (peptide D) and aa 256-275 (peptide B) sequences (Table 3; panel B). All the discrepances between the two sequences are probably due to ambiguities in the protein microsequencing. This result suggests that *hsdr* is very likely the gene coding for PS-68 protein or a spliced form of it although we cannot completely refuse the hypothesis that they are two different genes belonging to the same family.

2. The region from aa 53 to aa 175 shows 49.5% identity and 58.5% similarity with the sequence from aa 24 to aa 146 of the

1. human sdr deduced protein sequence
2. mouse sdr deduced protein sequence

A:

```
Identity      = 82%
Similarity    = 85%


          10        20        30        40        50        60        70
1.  MGEDAAQAEKFQHPGSDMRQEKPSSPSPMPSSTPSPSLNLGNTEEAIRDNSQVNAVTVLTLLDKLVNMLDAV
    ||||||||||||||  || |||||||||||||||||||||| |||||||||||||||| |||||||||||||
2.  MGEDAAQAEKFQHPNTDMLQEKPSSPSPMPSSTPSPSLNLGSTEEAIRDNSQVNAVTVHTLLDKLVNMLDAV
          10        20        30        40        50        60        70

          80        90       100       110       120       130       140
    QENQHKMEQRQISLEGSVKGIQNDLTKLSKYQASTSNTVSKLLEKSRKVSAHTRAVKERMDRQCAQVKRLEN
     |||| |||||| |||||||||||||||||||||||||||||||||||||||||||:||::||| |||||||
    RENQHNMEQRQINLEGSVKGIQNDLTKLSKYQASTSNTVSKLLEKSRKVSAHTRAVRERLERQCVQVKRLEN
          80        90       100       110       120       130       140

       150       160       170       180       190       200       210
    NHAQLLRRNHFKVLIFQEENEIPASVFVKQPVSGAVEGKEELPDENKSLEETLHTVDLSSDDDLPHDEEALE
    |||||||||||||||||| ||||||||| ||  | |||||| |||||||||| |||||||:|| ||||||
    NHAQLLRRNHFKVLIFQEESEIPASVFVKEPVPSAAEGKEELADENKSLEETLHNVDLSSDDELPRDEEALE
       150       160       170       180       190       200       210

      220       230       240       250       260       270       280
    DSAEEKVEESRAEKIKRSSLKKVDSLKKAFSRQNIEKKMNKLGTKIVSVERREKIKKSLTSNHQKISSGKSS
    |||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||| |||| ||||||
    DSAEEKMEESRAEKIKRSSLKKVDSLKKAFSRQNIEKKMNKLGTKIVSVERREKIKKSLTPNHQKASSGKSS
      220       230       240       250       260       270       280

     290       300       310       320       330       340       350       360
    PFKVSPLTFGRKKVREGESHAENETKSEDLPSSEQMPNDQEEESFAEGHSEASLASALVEGEIAEEAAEKAT
    ||||||| ||||||||||| |||||| ||      ||  | || || || ||||| |:| ||  : | |||:
    PFKVSPLSFGRKKVREGESSVENETKLED-----QMQEDREEGSFTEGLSEASLPSGLMEG--SAEDAEKSA
     290       300       310            320       330       340       350

          370       380       390       400       410       420
    SRGSNSGMDSNIDLTIVEDEEEESVALEQAQKVRYEGSYALTSEEAERSDGDPVQPAVLQVHQTS
     || ||: || |||| |||||| ||| ||| ||||  | | ||| |        |||||| | ||:
    RRGNNSAVGSNADLTIEEDEEEEPVALQQAQQVRYESGYMLNSEEMEEPSEKQVQPAVLHVDQTA
        360       370       380       390       400       410
```

1. human sdr deduced protein sequence
2. aminoacids sequence of the CN-Br cleavage fragments of PS-p68 protein

B:

```
        X         90        100         X
1.      EQRQISLEGSVKGIQNDLTKLSKYQASTSN
        |||||||||||||||||||||||||||| |
pepA    EQRQISLEGSVKGIQNDLTKLSKYQASTXN
        X         10        20        30


        X    140       150       X
1.      DRQCAQVKRLENNHAQLLRRNHFKV
        ||| ||||||||| ||||||| |||
pepD    DRQXAQVKRLENNXAQLLRRNXFKV
        X         10        20   X


        X 260       270     X
1.      NKLGTKIVSVERREKIKKSL
        ||||||||||||||||| ||
pepB    NKLGTKIVSVERREKIKXSL
        X         10        20
```

1. human sdr deduced protein sequence (53-175)
2. activated c-raf clone 7N-1

C:

```
Identity      = 49%
Similarity    = 58.5%


          60        70        80        90       100       110       120
1.  VNAVTVLTLLDKLVNMLDAVQENQHKMEQRQISLEGSVKGIQNDLTKLSKYQASTSNTVSKLLEKSRKVSAH
    | |||| |||:|| ||:     | |  :  ||  | |||: ||  |  ||:     |||| : || |: :||:|
2.  VHAVTVVTLLEKLASMLETLRERQGGLARRQGGLAGSVRRIQSGLGALSRSHDTTSNTLAQLLAKAERVSSH
         30        40        50        60        70        80        90

       130       140       150       160       170
    TRAVKERMDRQCAQVKRLENNHAQLLRRNHFKVLIFQEENEIPASVFVKQP
     |  ||  |  ||| ||| ||: |  |      || | || |:||| | | |
    ANAAQERAVRRAAQVQRLEANHGLLVARGKLHVLLFKEEGEVPASAFQKAP
      100       110       120       130       140
```

human 7N-1 clone, which is an activated form of the c-raf gene (Table 3; panel C). The first 568 aminoacids at the N-terminus of this oncogenic fusion protein is formed by a previously undescribed human cellular gene. This homology could suggest that *sdr* is a member of a new gene family (177).

1.13 *hsdr* mapping in the human genome.

In order to clone the *sdr* human gene, 1000000 plaques of a human genomic library were screened using a 300 bp fragment of the 5' end of the coding region of *hsdr* cDNA. One clone was able to cross-hybridize with our probe several times. After subsequent restriction mapping analysis, it resulted to contain a 12 Kb fragment of the human *sdr* gene.

In collaboration with Prof. G. Della Valle (Dipartimento di Genetica e Microbiologia, Universita' di Pavia), the *hsdr* gene was mapped in the human genome. The '*in situ* hybridization' experiment localized the *hsdr* gene on the human chromosome 2q32. This part of genome is very well known because 2q32.1 and 2q33 are regions commonly identified as 'fragile sites' (161). These are specific regions of chromosomes where the DNA either has failed to package for mitosis or has prematurely despiralized. They are classified according to their frequency and the conditions of tissue culture under which they are expressed. These two fragile sites are very common and are induced by aphidicolin treatment. The only fragile site of clinical significance is the rare one at Xq27.3 associated at the mental retardation pathology known as fragile-X syndrome. The other fragile sites known have not yet been described to correlate with

Human Chromosome 2

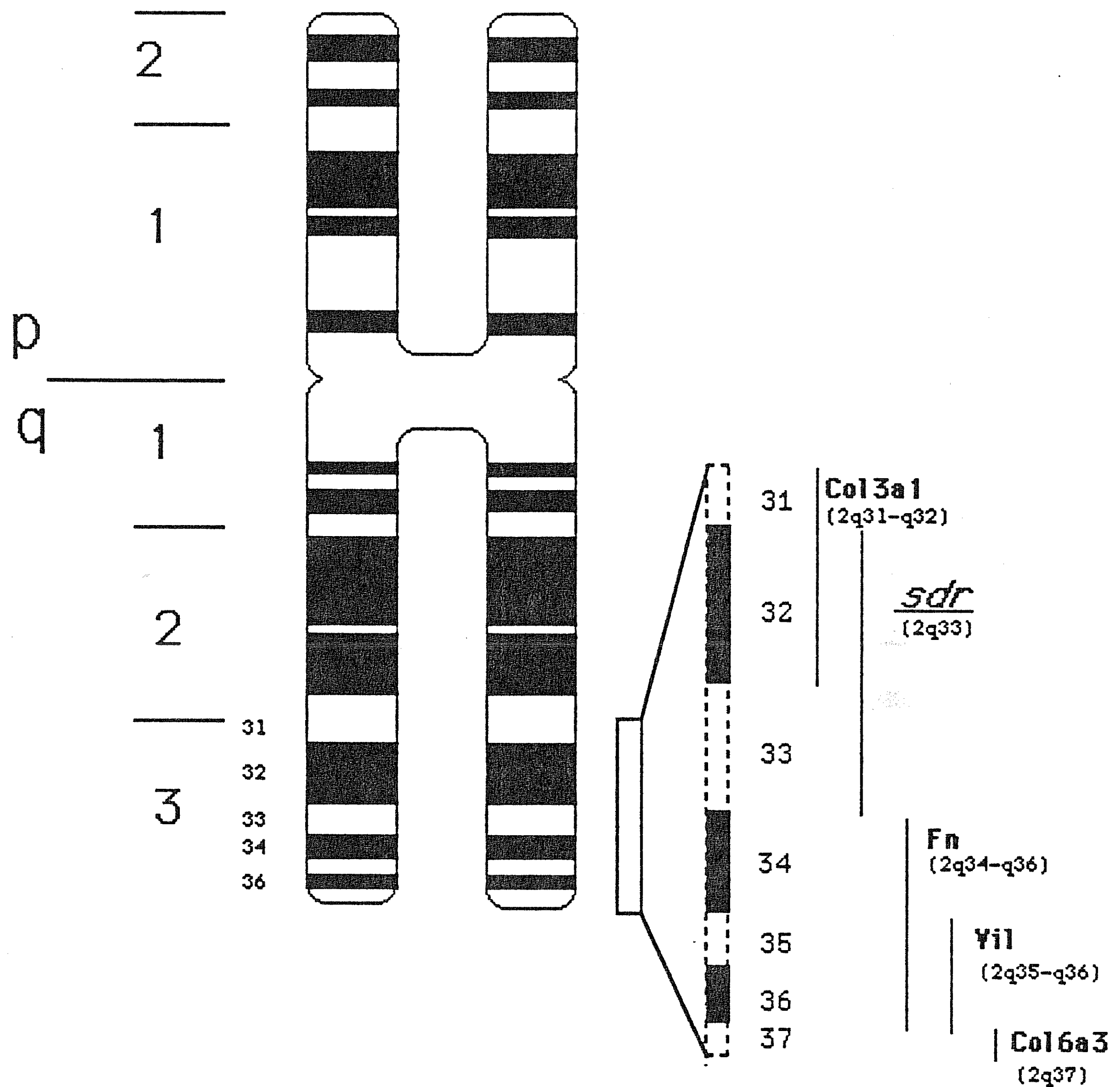


Table 4:

A schematic rapresentation of a portion of human chromosome 2. The enlarged region is synthenic to a part of the murine chromosome 1.

genetic pathologies, however they have been discussed as possibly having some role in oncogenesis or as points at which chromosomes break form constitutional rearrangements.

Moreover this part of genome is very well known because there is a perfect synthenic homology between mouse and humans (Table 4). Infact the region between the two loci Col3a1 and Col6a3 that maps at the proximal part of murine chromosome 1 is conserved in the long arm of human chromosome 2 from 2q31 (where Col3a1 gene maps) to 2q37 (where Col6a3 gene is localized). It has been previously shown that the order of the loci within these two markers is perfectly maintained. This apect has been absolutely confirmed by the *sdr* mapping data.

1.14 *hsdr* mRNA levels under different growth conditions.

I investigated the level of *hsdr* gene expression during different growth conditions by performing some Northern blot analysis. I studied the expression of *hsdr* in growing and resting human fibroblasts particularly referring to its expression during different growth arrested conditions. In order to induce the quiescent state by contact inhibition and by serum starvation, IMR90 cells were seeded in 10% FCS and subsequently the medium was changed every two days with fresh 10% for 8 days (density dependent growth inhibition) or with 0.5% FCS for 3 days (serum starvation). The *hsdr* probe recognizes a 3 Kb long transcript that is present in growing fibroblasts, up regulated in serum deprived cells but uninduced in density dependent growth inhibited cells (Fig. 9; Panel A). Total RNA loaded on the agarose gel for each

condition was normalized by probing the same filter with *gapdh* cDNA. The percentage of cells in S phase was measured by BrdU incorporation.

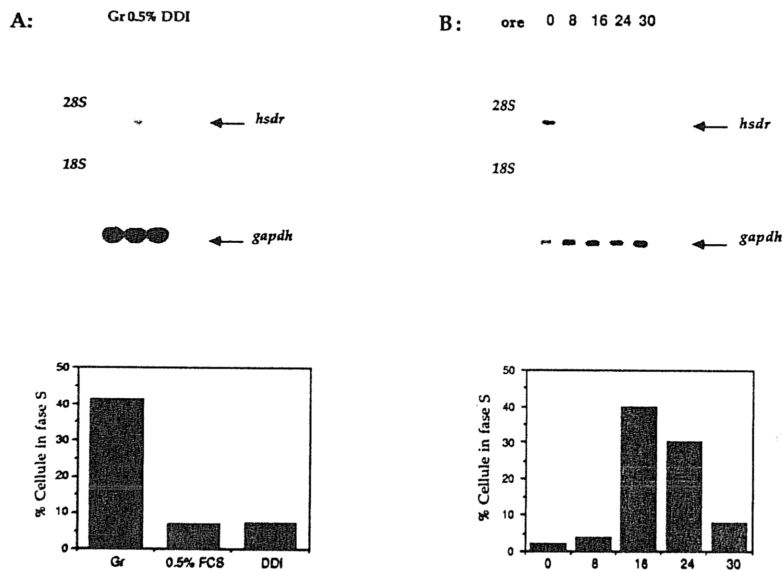


Fig. 9. Analysis of *hsdr* mRNA level during growth arrest induction and reentry into the cell cycle.

Northern blot analysis was performed on equal amounts of total RNA (10 µg) isolated from panel A) growing IMR90 cells, cells after 72 h from 0.5% FCS addition and density dependent inihibited cells panel B) IMR90 cells after 72 h from 0.5% FCS addition and at the indicated times after addition of 20% FCS. The same blot was probed, as indicated, with *hsdr* and *gapdh* cDNAs. The analysis of DNA synthesis levels for each time course is shown at the bottom.

Then I investigated the *hsdr* mRNA expression during the reinduction into the cell cycle. Total RNA was extracted from serum starved IMR90 fibroblasts or after various time of a sinchronous induction into cell division with 20% FCS. The highest transcript level was observed in quiescent unstimulated fibroblasts. *hsdr* mRNA reached the lowest level 8 hours after 20% serum addition and then slightly increased at 16 hours. Later it decreased again significantly at 30 hours (Fig. 9; Panel B). RNA amounts were normalized hybridyzing the same Northern blot with *gapdh* cDNA. The percentage of cells entering S phase for each time after 20% serum addition is also shown.

The expression of *hsdr* RNA confirms the previously described regulation of its murine homologue in NIH3T3 fibroblasts.

1.15 Production and specificity of rabbit antisera.

In order to analyze the protein product of *sdr* gene a rabbit was immunized with the bacterially expressed glutathione transferase Sdr fusion protein (GST-Sdr).

The murine cDNA lacking the first 112 aa was cloned in a pGEX-3X expression vector at the C-terminus of the glutathione transferase gene. The GST-Sdr protein was partially purified on Sepharose glutathione beads, separated by SDS-PAGE and injected into the rabbit after electroelution from gels. Around 100 µg were introduced in each injection. The rabbit antiserum was purified on a GST-Sdr affinity column and then tested for specificity.

The human cDNA was transcribed directly in the λGDST3/T7 vector using the T7 RNA Polymerase. In vitro capped mRNA was translated in a cell free system containing ^{35}S methionine and after immunoprecipitation the reaction products were analyzed by SDS PAGE (Fig. 10; lane a). The antibody was also used to immunoprecipitate Sdr in whole ^{35}S methionine labeled extracts of growing IMR90 cells (Fig. 10; lane b)

In labeled cellular lysates the antiserum recognizes a very closely comigrating 70-75 kd doublet that has the same apparent size as the primary *in vitro* translation product. Interestingly it can be observed that the relative migrating size of Sdr in SDS PAGE is greater than the one predicted from the aminoacid sequence analysis. The reason of this difference is not known but it could be related to the high charge of the protein (21% acidic residues) or to the relatively high content of proline (17 residues).

1.16 Sdr antisera recognizes a 75 Kd phosphoprotein.

Many comigrating forms has been described to result from postranslational modifications of the same protein product. Thus I tested if the difference in electrophoretic mobility found in growing cells could represent a modification of the phosphorylation state of Sdr.

IMR90 cells were labelled either with ^{35}S methionine for 4 hrs or ^{32}Pi for 12 hrs in precursor free DMEM medium prior the lysis. The labeled lysates were immunoprecipitated with anti Sdr or control antibodies (data not shown) and the immunocomplexes

analyzed by 7.5% gel electrophoresis. This experiment demonstrates that the immune purified antibody recognizes the 70/75 Kd protein while in ^{32}P lysates Sdr appears to be labeled only in the slower migrating form (Fig. 10; lane e and f).

Since Sdr is a phosphorylated protein, I tested whether the amount of the slower migrating form of Sdr found in IMR90 cells can be reduced by phosphatase treatment. The ^{35}S methionine labeled immunocomplexes were divided into equal portions and either treated with bacterial alkaline phosphatase (BAP) or left untreated prior to electrophoresis. Fig. 10; lane c and d shows that, after phosphatase treatment, the doublet found in the ^{35}S labeled fibroblasts is converted to the lower migrating form.

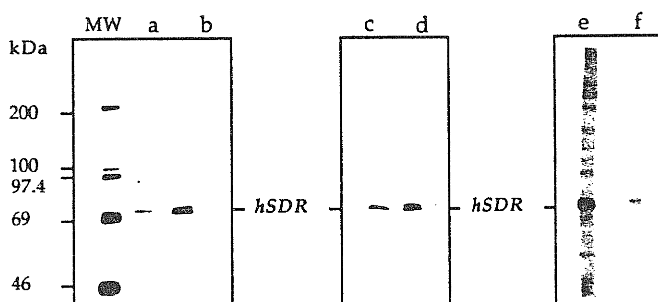


Fig. 10: Analysis of *hsdr* protein product:

Immunoprecipitation with anti SDR of a) *in vitro* translated products of *hsdr* cDNA, b) ^{35}S labeled lysates of IMR90 growing cells, c) ^{35}S labeled lysates treated *in vitro* with phosphatase, d) ^{35}S labeled lysates not treated with phosphatase e) ^{35}S labeled lysates of IMR90 growing cells f) ^{32} P labeled lysates of IMR90 growing cells

1.17 Sdr protein neo-synthesis in different growth conditions.

The pattern of *hsdr* mRNA expression during growth arrest has been previously analyzed (see fig. 9). To determine whether the Sdr protein was similarly regulated, the active biosynthesis of *hsdr* product in IMR90 cells was analyzed.

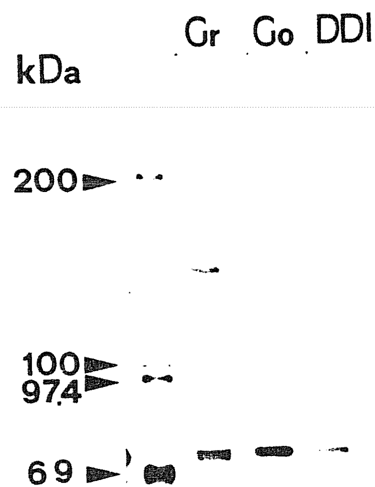


Fig. 11: Analysis of Sdr protein neo-synthesis.

Immunoprecipitation of ^{35}S labeled lysates with anti SDR of Gr) growing IMR90 cells, Go) cells starved for 72 hours in 0.5% FCS, DDI) density dependent inhibited cells.

Equal amounts of TCA precipitable proteins from growing, growth arrested by serum starvation or contact inhibition ^{35}S methionine labeled cells were immunopreciptated and the complexes separated by gel electrophoresis.

In growing cells Sdr is present as the previously described 70/75 kd doublet, it becomes upregulated in serum starved growth arrested cells as a single band (the slower migrating form), while in density dependent arrested cells only the slower form is expressed at a basal level.

From the figure 11 it can be deduced that the biosynthesis of Sdr protein follows the mRNA regulation in the different quiescent states. Moreover It can be noted that in growing fibroblasts Sdr is expressed as a doublet while in quiescent cells only the phosphorylated state is present.

1.18 Sdr protein levels in different growth conditions.

To study the steady-state expression of Sdr protein during different growth conditions, Western immunoblotting analysis was performed. Fig. 12; panel B shows the level of Sdr protein in growing, serum starved and density dependent inhibited cells. Sdr is induced in serum starved cells with respect to growing cells but surprisingly contact inhibited growth arrested cells present much more total protein than cells in all other growth conditions analyzed.

To assess the expression of Sdr during Go->S transition, serum deprived IMR90 cells were reintroduced into the growth cycle by adding 20% FCS. Sdr level is transiently decreased after

4 hours from serum addition, then remains constant at a higher level until 24 hours when it is strongly and definitively down-regulated (Fig. 12; panel A). Equal amounts of extracted proteins were loaded for each time point as assessed by Comassie blu staining of separate gels. DNA synthesis was checked by BrdU incorporation in both the experiments.

EXPRESSION OF hSDR DURING DIFFERENT GROWTH CONDITIONS

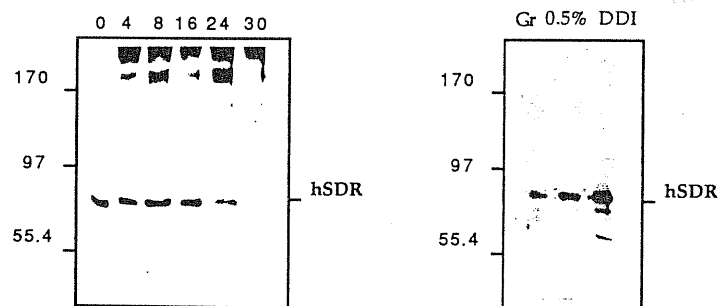
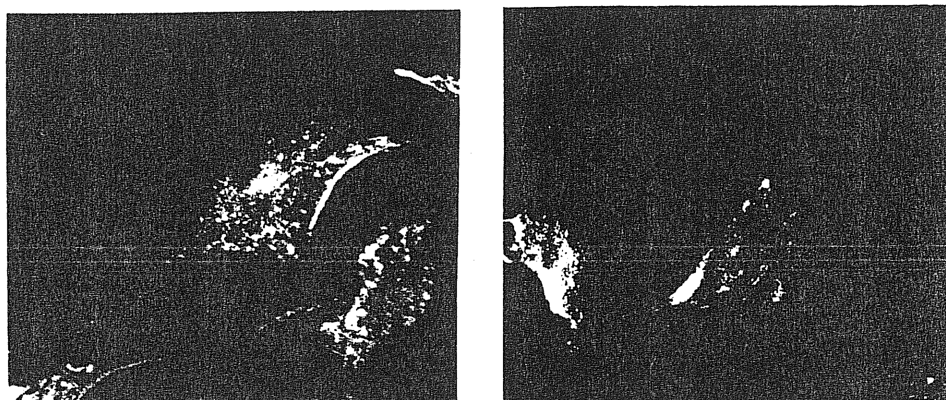


Fig. 12:

Western analysis using anti SDR of cellular lisates extracted from: on the left) IMR90 cells cultivated for 72 hours in 0.5% FCS and at different times from the addition of 20% FCS, on the right) IMR90 cells in growing state (Gr), 72 hours in 0.5% FCS (0.5%) and density dependent growth arrested (DDI).

1.19 Protein localization.

The affinity purified antibodies were used for indirect immunofluorescence localization of the 70/75 kd protein in IMR90 fibroblasts. In starved cells the antibody against Sdr displays a patchy uniforme cytoplasmic distribution with more evident spots. Interestingly in some cells Sdr antisera reveals a regular distribution localized on the lower side of the membrane and at the edge of the fibroblasts: it sometimes appears organized into linear arrays (Fig. 13; left).



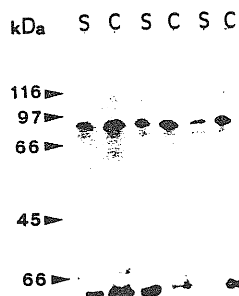


Fig. 13: Sdr subcellular localization.

top) left, immunofluorescence analysis on serum starved IMR90 cells using anti SDR antibodies; right, the same experiment made on pre-extracted cell with PMG2 buffer; bottom) immunoblotting of soluble (S) and cytoskeleton (C) fractions from cells untreated, treated with taxol and nocodazole. The same extracts were probed with an anti tubulin.

Not all the cells were equally stained with the Sdr antibody; some of them expressed Sdr at a higher level while others were barely decorated (data not shown).

Detergent extraction of cultured cells with PM2G buffer define a "cytoskeletal preparation" containing micro, macro and intermediate filaments. We have found that the Sdr patchy labelling is resistant to PM2G buffer extraction before fixation suggesting that the antigen is linked to the cytoskeletal network (Fig. 13; right). Interestingly preextraction of cells led to a clearer staining of the cytoskeletal preparates, suggesting that there may also be a significant soluble pool of protein as confirmed below by cellular fractionation.

In separate experiments, epithelial COS7 cells were transfected with an eukaryotic expression vector containing the whole coding region of *hsdr.* While these cells are not normally reactive with Sdr antisera, the transfected cells display the same Sdr detergent resistant distribution as previously described (data not shown).

1.20 Double immunofluorescence localization with cytoskeletal elements.

We performed double immunofluorescence microscopy to relate the Sdr localization with each component of the cytoskeletal network within the same cell.

Human serum deprived fibroblasts were labelled in separate experiments with Phalloidin-Texas red and with monoclonal antibodies anti vimentin or tubulin. While double staining with microfilaments led to complete different patterns (data not

shown), double labelling with antibodies against intermediate or macrofilaments shows overlapping areas of staining, suggesting a possible interaction of the Sdr with such cytoskeletal elements (Fig. 14). Althought many spots were aligned in a pattern consistent with a colocalization with microtubules, there is also some labelling not associated with this structure. Moreover no reactivity of Sdr antibodies with mitotic spindle or mid bodies was apparent during mitosis (data not shown). Similar distribution were found following alternative fixation procedures (e.g. 3% paraformaldheide, with or without detergent preextraction; data not shown)

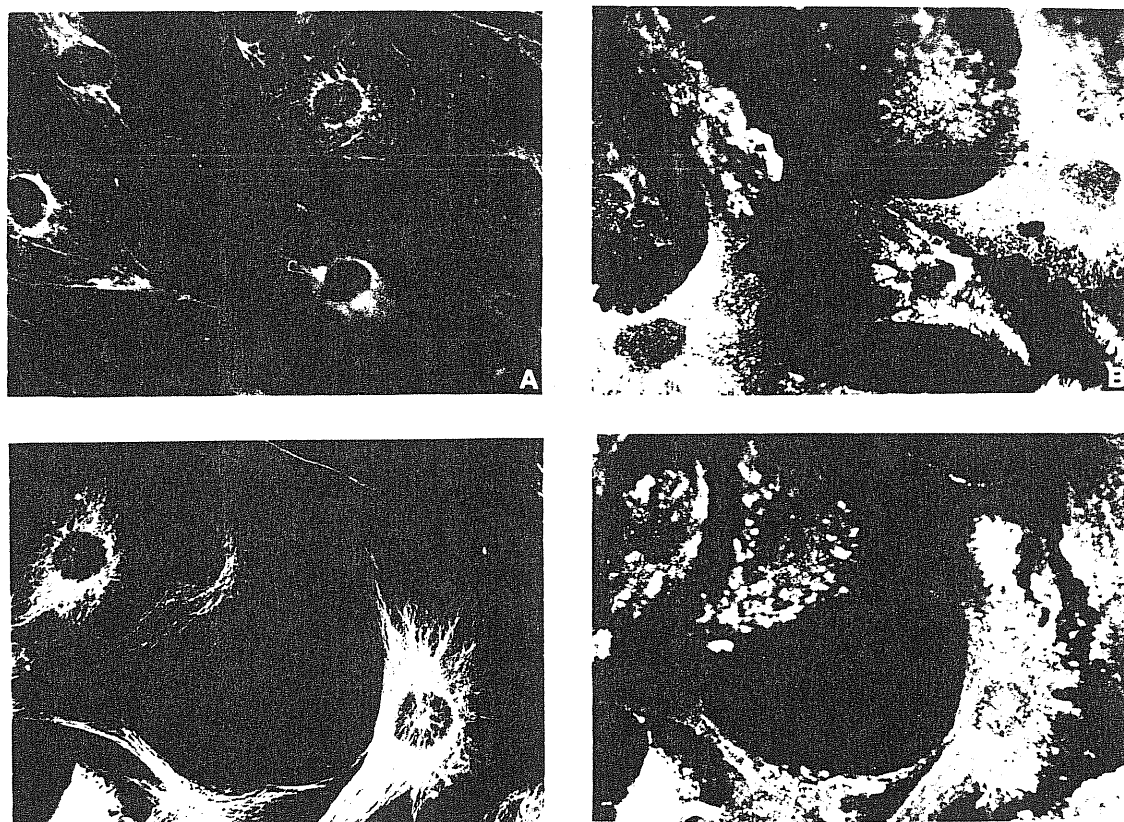


Fig. 14:

Double immunofluorescence analysis on serum starved cells with: B and D using policlonal antibodies anti Sdr; A anti vimentin and C anti tubulin.

1.21 The effect of drugs on Sdr localization: immunofluorescence analysis.

The localization of Sdr protein in serum deprived fibroblasts strongly suggests an in vivo colocalization or interaction with the macrofilament and intermediate networks.

Therefore the effects of microtubule active drugs such taxol and nocodazole on Sdr distribution was investigated. Nocodazole stimulates microtubule disassembly while taxol enforces assembly of total cellular tubulin. Depolimerization of the interphase microtubules with 10 µM nocodazole distrupts the punctate cytoplasmic pattern characteristic of untreated cells. The antigen does not become diffuse as does tubulin but appears to collapse from the edges of the cell to apical/nuclear region of the fibroblast.

Intermediate filaments are known to colocalyze and interact with microtubules, and their organization is also affected by nocodazole treatment. A primary association of Sdr with intermediate filaments is unlikely since a short term nocodazole treatment, which also distrupts the patchy organization of Sdr, does not affect significantly the intermediate filament network (Fig. 15).

Then the effects of a forced microtubular assembly were analyzed in relation to Sdr localization. Treatment of serum deprived fibroblasts with taxol during repolimerization of microtubules after removal of nocodazole, induces formation of random short tubulin polimers in the cytoplasm. These reorganized microtubules are strongly labeled by Sdr antisera

althought there is also significant cytoplasmic staining which does not appear to be microtubule associated.

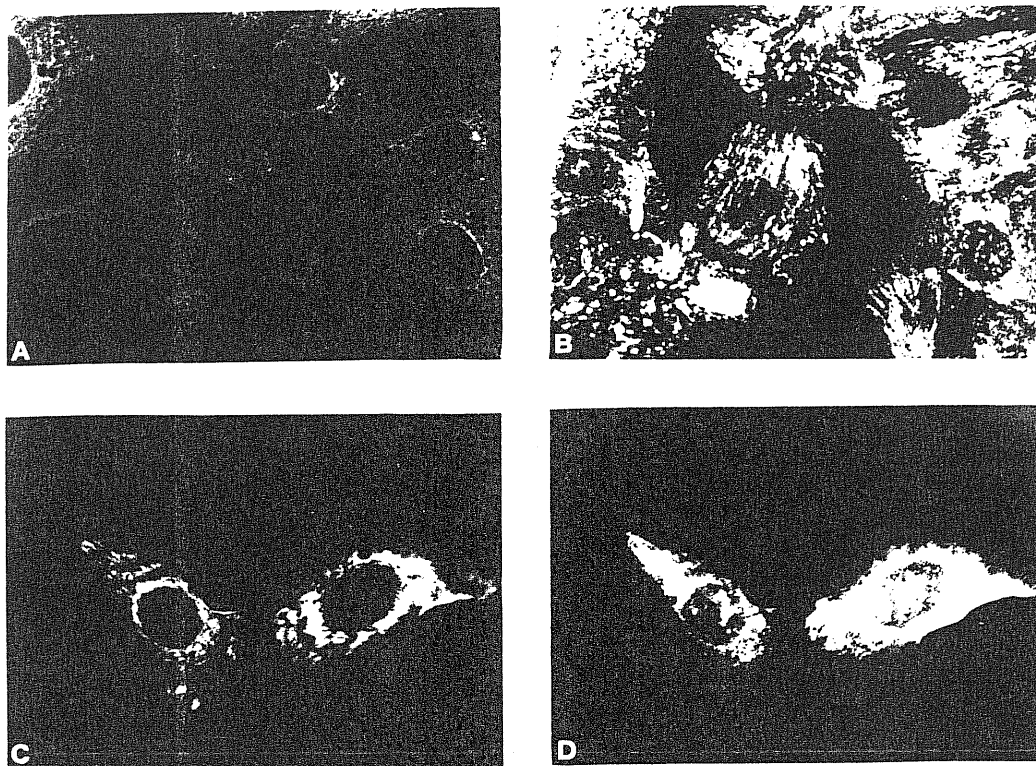


Fig. 15:

Double immunofluorescence analysis on serum starved cells treated with nocodazole plus taxol with: B and D using policlonal antibodies anti Sdr; A anti vimentin and C anti tubulin.

1.22 The effect of drugs on Sdr localization: biochemical analysis.

Since Sdr localization appears to be resistant to detergent extraction but affected by addition of drugs that are able to modify the macro- and intermediate filaments organizations, the Sdr association with cytoskeleton was further analyzed by immunoblotting soluble and cytoskeletal fractions of serum deprived and drugs-treated cells.

Serum deprived growth arrested cells were incubated for 12 hours with 10 µM taxol or 10 µM nocodazole and then processed as described in materials and methods. Equal amounts of total soluble or cytoskeletal proteins were loaded as assessed by separate commassie stained gels. The immunoblots were decorated with Sdr antibody or with a monoclonal antibody against alpha tubulin as control. As shown in Fig. 13 Sdr protein *in vivo* is present both in the soluble and cytoskeletal fractions; the cytoskeletal detergent insoluble level was apparently not affected by nocodazole depolimerization of microtubules or by taxol stimulated tubulin polimerization.

2. Effects of wild type and mutated *c-myc* ectopic expression on the cellular ability to enter into S phase.

2.1 Introduction.

Recently it has become clear that Myc drives two coupled functions: proliferation and programmed cell death. Since successful proliferation in normal cells requires the active suppression of programmed cell death, it has been postulated that c-myc can provide the first signal leading either to apoptosis or to cycle progression, and growth factors may provide a second signal to inhibit apoptosis and allow c-myc to drive cells into cell cycle. Several papers have focussed on the c-myc ability to activate the entry into S phase but the results have been contradictory. Moreover a lot of work has been done on the dissection of c-myc domains involved in different properties. The purpose of this study was to definitively demontrate the c-myc ability to drive a quiescent cells into the S phase once the apoptotic pathway was inhibited. I also wanted to analyze what domains of c-myc are involved in this process in order to relate this property to the other function of c-myc (S. Goruppi at al., manuscript in preparation).

2.2 Suppression of the apoptotic pathway.

In order to suppress the programmed cell death induced by Myc overexpression in quiescent cells, NIH3T3 cells transformed

by v-myc were used as a model system. As reported by G. Evans et al. fibroblasts overexpressing c-myc protein in low serum die by programmed cell death (52). An apoptotic cell in culture can be identified by two characteristic phenotypes: at the microscope there is a visible loss of cell to cell contact, nuclear condensation and membrane blebbing; at the molecular level the DNA from dying cells reveals fragmentation of chromatin into nucleosome ladders. Considering these two aspects as potential assays in the analysis, v-myc transformed NIH3T3 cells were cultivated both in 10% FCS and in 0.5% FCS in order to have cultures of both living and dying cells. The purpose was to obtain a quiescent culture where the ectopic expression of c-myc is not able to induce apoptosis. Since PMA was already shown to inhibit spontaneous DNA fragmentation caused by different stimuli in chronic lymphocytic leukemia lymphocytes, the ability of this molecule to elicit the same effect in the above described system was tested (54). The results of this analysis is shown in figure 16. The microphotographs by the phase contrast microscopy show that the v-myc NIH3T3 cells cultured both in 10% FCS and 0.5% FCS plus PMA are perfectly lifeful while dying cells are visible in the dish containing only 0.5% FCS. Genomic DNA was extracted from the three culture dishes according to the method described in S. Gustincich et al. and 2 μg of uncut DNA were loaded on a 0.7% agarose gel. While genomic DNAs extracted from cells cultured in 10% FCS or in 0.5% FCS plus PMA are undegraded, the DNA extracted from cells cultured in 0.5% FCS shows the characteristic degradation pattern of apoptosis.

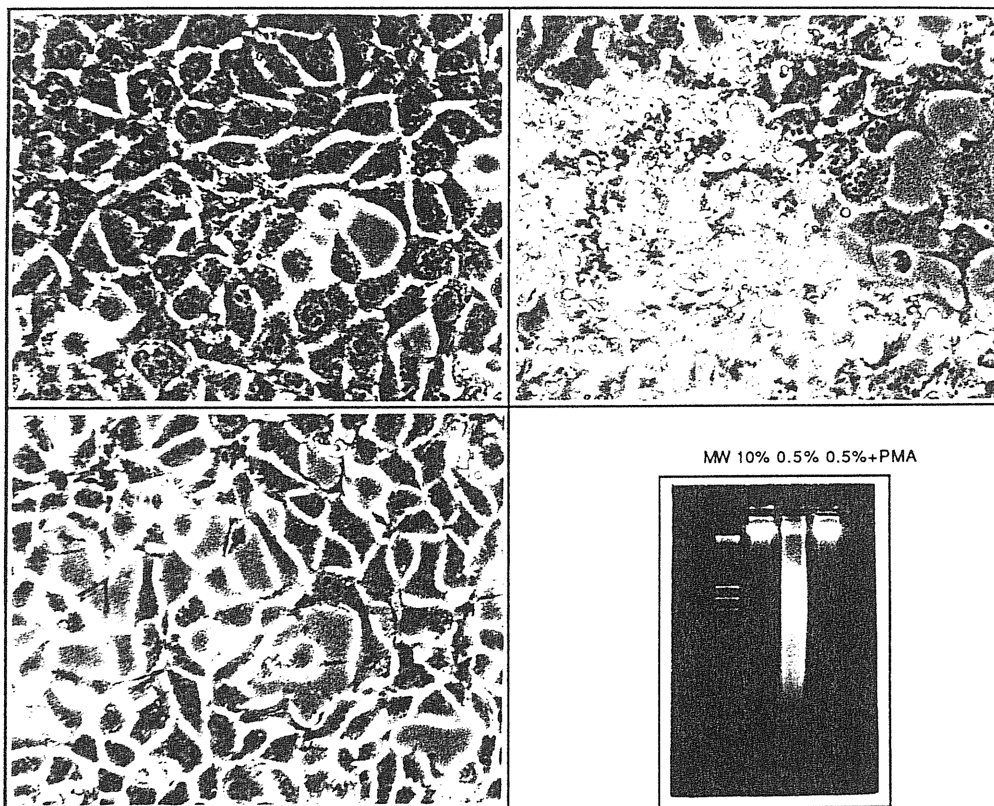


Fig. 16: Analysis of the apoptotic state of v-myc transfromed cells cultured in different growth conditions.

On the top/left: cells cultured in 10% FCS; on the top/right: cells cultured in 0.5% FCS; on the bottom/left: cell cultured in 0.5% FCS plus PMA; on the bottom/right: agarose gel electrophoresis of genomic DNA extracted from these three cellular conditions.

2.3 Microinjection of wild-type and mutant c-myc in serum starved NIH3T3 cells in the presence of 10^{-7} PMA.

Since overexpression of v-myc in serum starved cells in the presence of PMA does not induce apoptosis, this system was used to assess if c-myc is indeed able to drive cells into growth cycle. The genomic human c-myc fragment cloned under the Moloney murine leukemia virus enhancer-promoter (gently provided by W. Lee, University of Pennsylvania) was used in these studies.

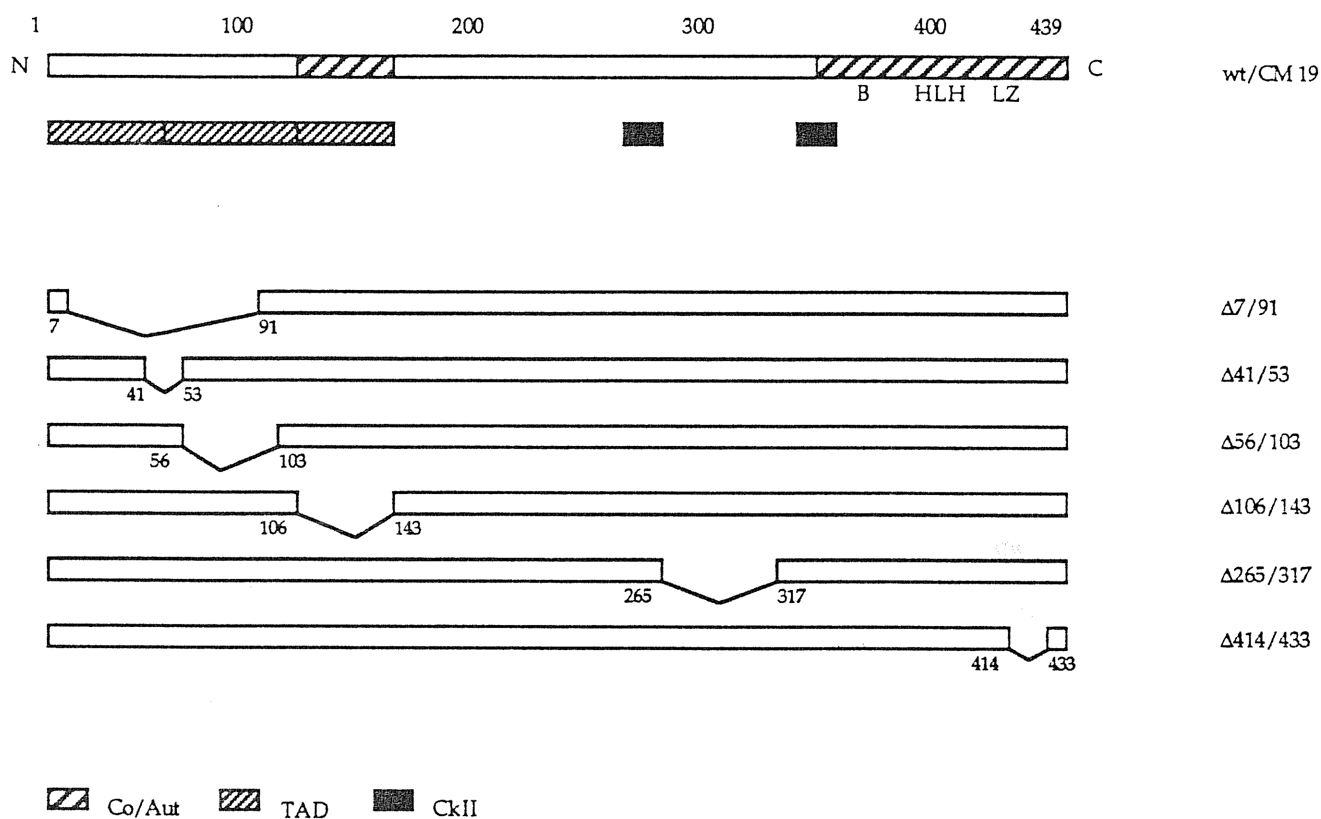


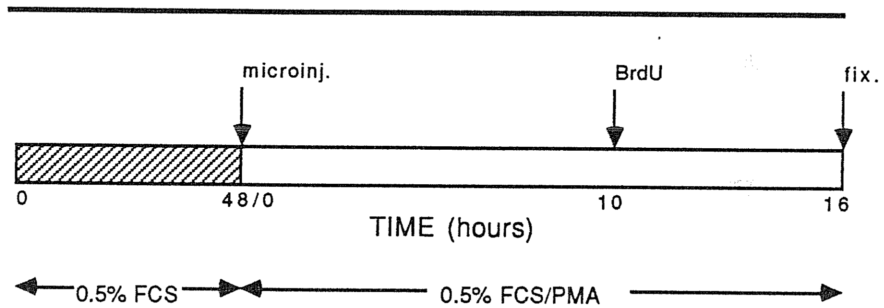
Table 5:

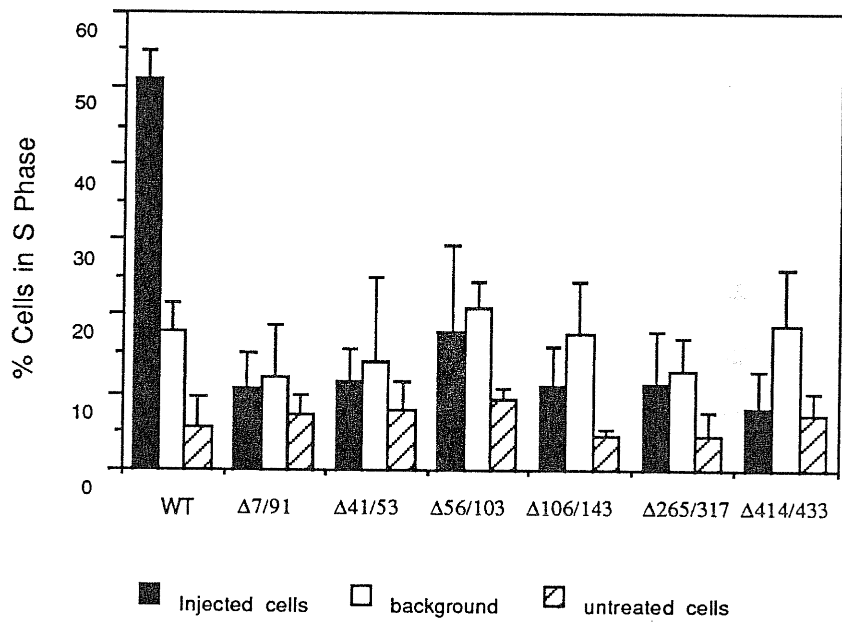
A schematic rapresentation of c-myc protein structure. Co/Aut: Cotransformation/Autosuppression; TAD: TransActivation Domain; CkII: phosphorylation site for Casien Kinase II.

NIH3T3 cells maintained for 48 hours in 0.5% FCS were microinjected in the nucleus with the wild-type (wt) c-myc containing plasmid at a concentration of 50 ng/μl. At the end of the microinjection, PMA was added to the medium at a final concentration of 10^{-7} M. After 10 hours BrdU was added at a final concentration of 50 μM and left for 16 hours when cells were fixed for the immunofluorescence analysis. Cells in S phase were immunostained with the monoclonal antibodies anti BrdU while the expressed products were recognized with the anti myc rabbit policlonal antibody (the antisera was provided by R. Eisenmann, F. Hutchinson Institute, Seattle, Washington and affinity purified by S. Goruppi). The average of the results obtained in 3 different experiments is shown in Table 6. 51% of the microinjected cells are found in S phase thus showing a clear growth activating property of c-myc.

At this point the role of different c-myc domains was analyzed. Among a large spectrum of c-myc mutants provided by W. Lee, six of them were chosen. Table 5 shows the position of the deletions in the c-myc protein structure. The use of mutants Δ7/91, Δ41/53 and Δ56/103 allowed us to dissect the N-terminus domain involved in the transactivation capacity of the wt protein. The region deleted in Δ106/143 has been shown to be absolutely required for all the c-myc activity while the Δ414/433 contains the LZ domain. Finally we chose the Δ265/317 mutant was used because it is differentialy dispensable in different assays. All the mutants have been automatically microinjected in the nucleus of starved cells in the same condition as wt: none of them were able to activate the entry in S phase.

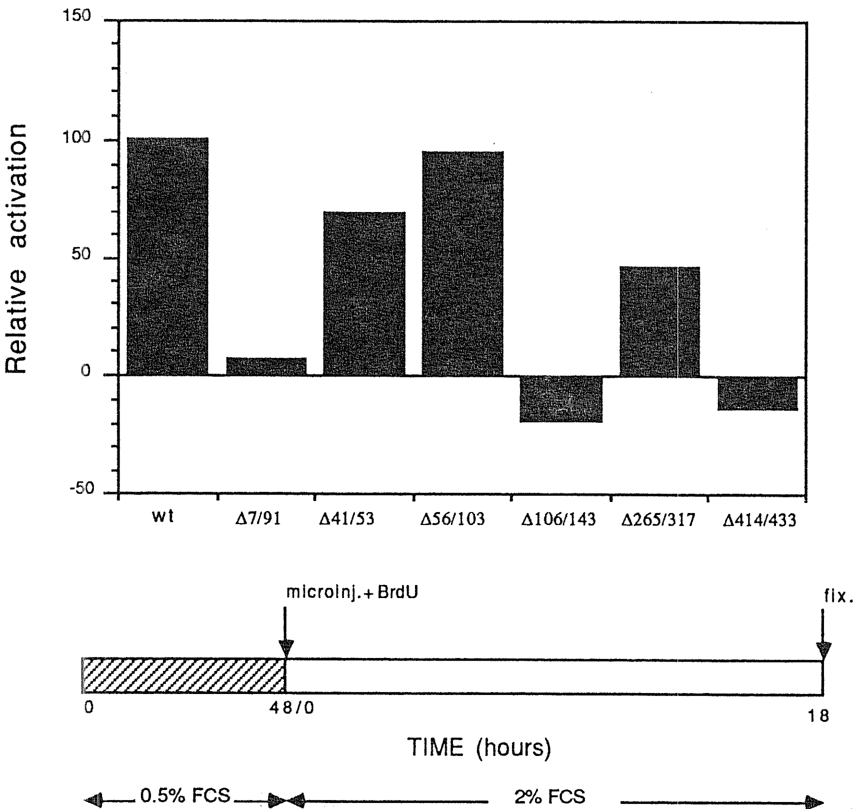
Injected DNA	Inj. cells analyzed	BrdU in inj. (%)	Brdu in uninj. (%)	Exp
ωτ	273	51.0 + 3.6	18.0 + 3.6	3
Δ7/91	322	10.6 + 4.5	12.3 + 6.6	3
Δ41/53	165	11.6 + 4.2	14.3 +10.7	3
Δ56/103	256	18.0 + 11.5	21.0 + 3.5	3
Δ106/143	273	11.0 + 5.2	17.7 + 6.8	3
Δ265/317	248	11.3 + 6.8	12.7 + 4.5	3
Δ414/433	372	8.3 + 4.5	18.7 + 7.5	3





2.4 Micronjection of wild-type and mutant c-myc in serum starved NIH3T3 and subsequently activation with serum.

Since J. Karn et al. showed that c-myc is able to regulate the cell cycle length by shortening the G1 period, microinjection experiments were performed in order to assess if c-myc is able to anticipate S phase entry with respect to the time required during 10% FCS activation. When arrested serum starved cells are stimulated with 10% FCS they normally enter into S at the beginning of the 12th hour. wt c-myc containing plasmid were microinjected into the nucleus of serum starved cells and the culture medium was changed with 10% FCS. After performing several experiments with different BrdU pulse labellings, no difference in the percentage of cells in S phase was noted between microinjected and background cells. Thus a different approach was tackled to analyze this problem. G. Evan et al. have recently demonstrated the ability of 2% FCS to suppress the apoptotic inducing properties of c-myc. In my laboratory serum starved cells are activated with 2% FCS to study the effects on G1 progression of total mRNA extracted from growing and resting cells (see below). When 2% FCS is added to a serum deprived culture, cells start to replicate DNA 12 hours later, which exactly corresponds to the time when cells activated by 20% FCS. However the number of cells that are able to respond to this mitogenic stimulation is considerable lower. Thus I wanted to analyze if c-myc ectopic expression is able to increase the percentage of cells in S phase after 2% FCS treatement (Table 7). BrdU was added at the same time as the 2% FCS and



Injected DNA	microinj. exp	Inj. cells analyzed	BrdU in inj. (%)	BrdU in uninj. (%)
ωτ	5	371	88.6 ± 6.2	38.8 ± 5.9
Δ7/91	5	287	45.0 ± 8.8	41.6 ± 7.5
Δ41/53	4	211	68.1 ± 7.1	36.0 ± 3.5
Δ56/103	4	247	80.3 ± 5.5	32.3 ± 6.6
Δ106/143	4	275	37.3 ± 9.4	49.3 ± 6.1
Δ265/317	4	323	48.8 ± 8.4	31.0 ± 7.6
Δ414/433	4	287	33.0 ± 16.5	38.3 ± 12.9

cells were fixed 18 hours after the microinjection. As shown in table 7, almost all the microinjected cells are BrdU positive demonstrating that the wt c-myc is able to strongly activate entry in S phase. Using this system, overexpression of the same mutants as above was analyzed. The results were completely different. While deletion of the whole N-terminus, of the fondamental region 106-143 and of LZ completely shut off the c-myc ability to drive cells into S phase, regions Δ56/103 and Δ41/53 result completely dispensable in this assay. Moreover c-myc protein lacking the region 265/371 seems to be still able to elicit its mitogenic effect albeit at a reduced level.

3. Cloning of positive regulators of G1 progression.

3.1 Introduction.

As described in a previous section, many data have been reported in the literature on the capacity of different mRNAs to affect positively or negatively the G1 progression of resting or postmitotic cycling cells. In this laboratory, M. E. Ruaro et al. have finely dissected the presence of growth inhibitory and activatory mRNAs in NIH3T3 cells as assayed for the capacity to alter the normal S phase entry process. Similarly to the previous reported data on c-myc ectopic expression, poly(A)+ fraction of serum starved and growing NIH3T3 cells have been automatically microinjected in quiescent cells. Cells were subsequently activated with 2% FCS in the presence of BdrU and after 18 hours fixed and scored for the number of cells in S phase with a monoclonal antibody anti BrdU. The microinjected cells were identified because the mRNA was coinjected in the cytoplasm with the fluorescent dye dextran. While the mRNAs extracted from quiescent cells inhibit S phase entry, the mRNA extracted from growing cells were able to act positively on this process.

The aim of this work was to clone the gene/s responsible for this activating property on the entry in S phase. I decided to approach this problem with the following strategy:

1. size-selection of the poly(A)+ fraction of RNA of growing cells with sucrose gradient sedimentation and microinjection of each fraction in order to determine the average size of the mRNA responsible for this effect

2. construction of an oriented cDNA library in the λGDST3/T7 vector with the poly(A)+ from the same aliquot that is able to elicit the required activity

3. construction of an enriched probe for growing specific mRNAs using a substractive hybridization method

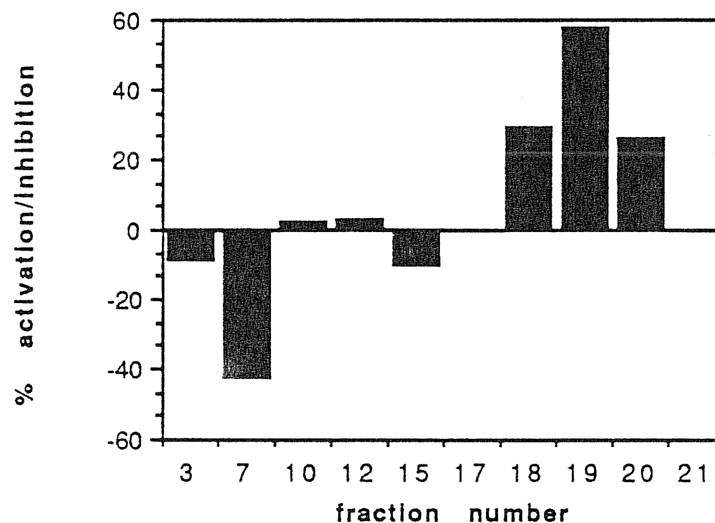
4. screening with the growing specific probe of the cDNA library of the size selected growing mRNAs.

5. analysis of the positive clones and subcloning in pGDSV7 plasmid to perform microinjection experiments

3.2 Size selection of poly(A)+ RNA and cDNA library construction.

I prepared 20 μg poly(A)+ mRNA from total RNA extracted from growing cells. Under growing conditions, 40% of cells are in S phase after one hour labelling with BrdU. An aliquot of this preparation was microinjected and assayed for its growth activating property. 10 μg of this mRNA were boiled for 1' in the presence of 0.1% SDS and immediately loaded on the sucrose gradient that was previously prepared using two stock solutions containing 20% and 40% of sucrose (see Materials and Methods). After 12 hours of ultracentrifugation at 2° C at 40000 r.p.m., 24 fraction of ~200 μl were collected from the bottom of the tube. Each fraction was precipitated in the presence of glycogen and resuspended in 20 μl of water. 1 μl aliquot was loaded on an RNA agarose gel to check the integrity of the mRNA and the quality of the size separation. Then 10 μl of each fraction were comicroinjected with dextran into quiescent NIH3T3 cells. After microinjection, cells were activated with 2% FCS in the presence

of BrdU and fixed 18 hours later for the immunofluorescence analysis. The results of this experiment are shown in table 8: fraction 19 has the capacity to activate S phase entry (60% relative activation) while fractions 18 and 20 present a lower relative activation level (30%). None of the remaining fractions had the same property. From the previously described data obtained from the electrophoretic analysis, fraction 19 contains mRNAs of 550 nt average size. I performed the same experiment starting from the poly(A)+ fraction of RNAs extracted from resting cells. In that conditions, 3% of cells were in S phase after one hour labelling with BrdU. An aliquot of this preparation was microinjected and assayed for its growth inhibiting property.



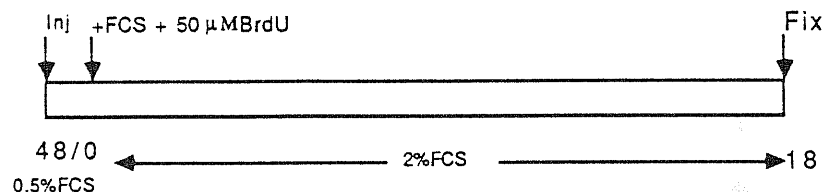


Table 8:

Description of the microinjection experiment with poly(A)+ fractions from sucrose gradient sedimentation.

10 µg of poly(A)+ RNA were fractioned by sucrose gradient sedimentation. 20 fractions were collected and microinjected as described above. Fraction containing mRNA up to 1000 bases had no effects on the S phase entry suggesting that the gene responsible for the growth activating capacity is differentially expressed in growing and resting cells. Thus I used the remaining content of the aliquot 19 of the 'growing' size as substrate for the synthesis of an oriented cDNA library cloned in the λGDST3/7. I chose this vector because it contains the same polilinker cassette of the pGDSV7 plasmid (G. Del Sal et al., submitted). In this polilinker there are 7 unique restriction sites and two different asymmetrical SfiI sites that allow the directional shuffling from lambda to the plasmid vector. Moreover in pGDSV7 the polilinker cassette is positioned 3' to the SV40 promoter to allow the transient expression of cloned cDNAs in mammalian cells. The EcoRI/HindIII oriented cDNA library contained $1x10^5$ clones. In order to check the quality of both the size selection and the library, I purified DNAs from 8 randomly chosen plaques. All the clones contained fragments of approximately 550 bp. These clones were sequenced using the T7 and T3 primers present in the λGDST3/T7 vector. The obtained sequences were utilized for a homology search in the EMBL Data Bank: four of them contained completely unknown genes while the remaining four clones code for different ribosomal proteins. Interestingly the known size of the mRNAs of these four genes was ranged from 450 to 650 bases.

3.3 Construction of the growing specific probe.

Starting from the observation that the growth activating gene is expressed only in growing cells, I decided to synthesize a growing specific probe to screen the cDNA library.

I used an aliquot of the poly(A)+ fraction of the RNAs extracted both from growing and resting cells to synthesize double stranded cDNAs. I tested different ratios between the amounts of the poly(A)+ and random primers to obtain cDNAs of an average size of 200 bp. The size was analyzed first by radioactively labelling the neo-synthesized cDNA and then performing a polyacrilamide gel electrophoresis. The optimal ratio was 1 µg of poly(A)+ and 10 µg of primers. Then I purified the blunted double stranded cDNAs on an A5 chromatography column. I was able to produce 5 µg of growing and 2 µg of resting cDNAs.

Four oligos were synthesized and purified on a denaturing 15% poliacrylammide gel. The oligos were the following:

MD179: CACAGGAGTTTGTCAGACTC

MD180: GAGTCTGACAAACTCCTGTG

OY82: TGAAGAGCTCTCGAGGAATTC

OY83: GAATTCCTCGAGAGCTC

5 µg of purified MD180 and OY83 were phosphorylated in the presence of ^{32}P γ-ATP and heated at 75° C for 10' in the presence of equimolar quantities of respectively MD179 and OY82 oligonucleotides. After this incubation, they were slowly cooled to room temperature to allow annealing and formation of the two oligovectors phosphorylated on one of their ends. The OY

oligovector contains restriction sites for EcoRI, XhoI and SacI enzymes.

1.5 μg of growing double stranded cDNAs of 200 bp average size were ligated both with 1.75 μg of MD oligovectors and with 1.5 μg of OY oligovectors, while 1.5 μg of resting cDNAs were ligated with 1.75 μg of MD olivectors. The ligation with the MD oligovectors was incubated at 16° C for only 4 hours to avoid the formation of linker concatamers of very large size. The products of the three ligations were then purified on a native 6% poliacrylammide gels to separate the unligated oligovectors from the cDNA-oligovectors molecules.

The oligonucleotides MD179 and MD180 were also prepared containing four biotins to the 5' end of the two molecules. The MD179-4B and MD180-4B oligos were purified first on a denaturing 15% poliacrylammide gel and then on a denaturing 20% poliacrylammide gel. This further purification step was absolutely required to eliminate the otherwise perfectly co-migrating not biotinilated forms of each oligos. Once purified, both the MD179-4B and MD180-4B were tested for their capacity to bind efficiently to streptavidin coupled to the paramagnetic beads (SA-PMP). Both the biotinilated oligos and the MD179 were kinased with ^{32}P γ-ATP and purified twice on a G-25 chromatograpy column. The labelled oligos were incubated 30' at room temperature with 300 μl of SA-PMP pre-washed three times in 0.5x SSC. Then the SA-PMP were separated with a magnete and analyzed for their radioactivity content at the scintillation counter. While no hot MD179 oligonucleotide was found to be bound to the SA-PMP, more than 97% of the hot MD179-4B and MD180-4B oligonucleotides were found to be retained by the SA-

PMPs demontrating that almost all the biotinilated oligos are able to bind the streptavidin.

At this point we used the MD179-4B, MD180-4B, OY82 and OY83 oligos as primers in the PCR amplification of the cDNA-oligovector molecules. I used 1/50th of the cDNA-oligovector purified preparation for each PCR reaction and tested the right amount of each couple of primers to obtain PCR products of 200 bp average size. Then I performed 10 amplification reaction of growing cDNA-MD in the presence of MD179-4B and MD180-4B oligos, 10 amplification reactions of arrested cDNA-MD in the presence of MD179-4B and MD180-4B oligos and 10 amplification reactions of growing cDNA-OY in the presence of OY82 and OY83 oligonucleotides. With the MD179-4B and MD180-4B primers I used the following amplification conditions: denaturation 94° C 30"; annealing 55° C 30"; extension 72° C 1' for 20 cycles. In the presence of OY 82 and OY83 primers the amplification conditions were: denaturation 94° C 30"; annealing 47° C 30"; extension 72° 1' for 4 cycles and denaturation 94° C 30"; annealing 57° C 30"; extension 72° C 1' for 20 cycles. In all the experiments one control PCR reaction has been perfomed in the absence of template. The PCR products were purified on a native 6% poliacrylammide gel in order to perfectly separate the not incorporated oligos from the growing cDNA-MD-4B, the growing cDNA-OY and the arrested cDNA-MD-4B.

These constituted the reagents to perform our substractive hybridization experiment. At first I tested the hybridization condition by using 30 µg of growing cDNA-MD-4B with 1 µg of hot growing cDNA-OY. The two cDNAs were pooled and precipitated in the presence of ammonium acetate. Then they were resuspended

in a solution containing 5 mM EDTA and SDS 0.1% and boiled for 2'. After adding 5 µl of hybridization buffer 5x (2 M sodium phosphate pH 6.8) the hybridization mixture was transferred into a glass capillary, sealed and submerged in boiling water. 5' later the capillary was transferred in a water bath at 55° C and left for 24 hours. At the end of hybridization, the capillary was cut and the content was transferred to an eppendorf tube where 80 µl of 0.5x SSC were added together with 100 µl of pre-washed SA-PMPs. After 30' of agitation at room temperature, the magnetic beads were captured by the magnete and the unbound solution was conserved. Under these conditions, I was able to substract 92.5% of the non-biotinilated hot cDNAs thus showing that this was due to the hybridization of non-biotinilated cDNAs to the same sequences present in the biotinilated form. I thus repeated this experiment hybridizing 30 µg of arrested cDNA-MD-4B with labelled growing cDNA-OY. I was able to substract 91.3% of the hot not biotinilated cDNAs. At the end of these two hybridization experiments I had two different growing cDNA-OY populations: one substracted with resting cDNAs and the other with growing cDNAs sequences as control. Considering that the amount of cDNA-OY recovered is very small and that contaminations from the MD-4B populations could occur (less than $1/300^{th}$ would be relevant), I thus decided to re-amplify with the OY oligonucleotides both substracted cDNAs populations. After testing the right ratio between the substrate ($1/10^{th}$ of the substracted cDNAs) and the OY primers to obtain PCR products of an average size of 200 bp, I performed two series of PCR amplifications. Every set of experiments included a control tube reaction without template. The PCR products were pooled and

purified on a native 6% poliacrylammide gel in order to eliminate the not incorporated OY primers.

In order to be sure that I enhriched for specific cDNAs present in the growing mRNA fraction, I cloned part of the growing specific probe using the SacI site present in the OY oligovector. Four clones were sequenced: while one clone codes for a yet unknown gene, the other three clones were particularly informative. One cDNA codes for the ST2 gene that has been previously described because it is highly induced after addition of mitogens in NIH3T3 cells and is known as a growing specific marker. The other two clones code for the ribosomal RNA genes. This is apparently in contradiction with the fact that I have synthesized the cDNAs from the poly(A)+ portion of the total RNA. However when cDNAs are primed using random esanucleotides the possible cloning of genes for ribosomal RNA is a very well described drawback. Moreover the cloned sequences were not randomly localized in the ribosomal RNA genes: while the total ribosomal RNA amount is increased 3-4 times in growing with respect to resting cells, the active transcription of ribosomal genes is induced more than 100 fold. The differences in ribosomal RNA content is thus the result of different regulations both at transcriptional and posttranscriptional levels. The two isolated sequences are not present in the final processed ribosomal RNA but are spliced out during the posttranscriptional processing. I have thus cloned the part of the rRNA that is highly induced in growing cells even if probably it has not been so much present in the cDNA. This result allowed me to think that I have obtained a very good enrichment for growing specific sequences.

3.4 Screening the size-selected growing mRNA cDNA library by using the growing specific probe.

I plated 10000 clones of the cDNA library of the size-selected mRNA on three different plates and transferred them to nylon filters twice for each plate. Both sets of filters were hybridized with two substracted probes: the growing cDNA-OY substracted with resting cDNA-MD-4B and the one substracted with growing cDNA-MD-4B as control. The two probes were labelled at high specific activity with the oligolabelling procedure. The unincorporporated nucleotides were removed by G-25 chromatography. The hybridization was performed in 10 ml of 1 M NaCl/1% SDS at 55° C in the presence of 100 µg/ml of salmon sperm DNA with both probes at a concentration of $1x10^{6}$ c.p.m./ml. After 24 hours, the filters were washed at 0.5x SSC/1% SDS at 60° C 30' twice. After three days exposure at -80° C, several differential signals were visible. More than 100 plaques were picked up and replated for the plaque purification procedure. Having observed that the average signals in the control experiment are less intense, I decided to synthesize hot cDNAs at a very high specific activity starting from the poly(A)+ fraction of resting cells RNA and use them as control probe in the plaque purification experiment. I could plaque purify 50 clones. After the hybridization with the hot cDNAs for resting cells and the growing specific probe, 33 clones were purified. A cross-hybridization analysis defined them as copies of only two different mRNAs.

Thus I have so far isolated two genes that have the expected size and are expressed specifically in growing cells. I subcloned

the two longest clones from the lambda vector into the plasmid pGDSV7 using the asymmetrical SfyI sites and sequenced them. Both genes resulted already cloned.

The clone 64 is known as the P198 gene. In vitro mutagen treatment of tumor cell lines generates at very high frequency stable immunogenic variants that are rejected by singenic mice. Since they fail to form tumors, these variants have been named *tum*$^-$, as opposed to the original *tum*$^+$ cells. P198 is the gene responsible for the *tum*$^-$ variant obtained after mutagenic treatment of P1, a clonal cell line derived from mouse mastocytoma P815. Nothing is known about the biological features nor the biochemical properties of the protein coded by this gene.

The clone 78 contains the cDNA for the ribosomal protein L29.

Work is now in progress in order to analyze the effects of the ectopic expression of these two clones on the S phase entry ability of murine fibroblasts. Moreover other positive signals at the primary screening are currently under investigation.

DISCUSSION

During the last years the most important and key regulatory genes of the cell cycle have been characterized in yeast. From the dissection of the G2/M transition control in *S. pombe* and of START in *S. cerevisiae*, a general model of the cell cycle machinery has been proposed. At both points, the formation of a multiprotein complex with kinase activity is the major control step; infact different stimuli deriving from many different sources (i. e. environmental conditions and presence of mating peptides) are transduced at this level and are able to interphere positively or negatively on SPF (S phase Promoting Factor) and MPF (Maturation Promoting Factor) formation. These two complexes contain two major components: one regulatory subunit named cyclin and one catalytic subunit named $p34^{cdc2}$. In these unicellular organisms the $p34^{cdc2}$ protein is present at both steps but with different partners. Cyclins generally are the targets of the regulatory pathways, infact the mating peptides that are able to inhibit the cycling of yeast cells act by suppressing the function of CLN2, one of the G1 cyclins in *S. cerevisiae*. Moreover the activation of the kinase activity depends also on many postranslational events that involve other kinases and phosphatases. The correct execution of the previous step in the cell cycle machinery program is tightly coupled to the initiation of the next one: these checkpoint controls are one of the most important features in the logic of cell cycle control.

In higher Eukaryotes the main characteristics of the organization of the cell cycle machinery are conserved. However a

large increase in complexity has been achieved for most of the cell cycle regulatory steps. This is probably due to a much more complex response to many different positive and negative signals. Thus many cyclins and cdc2/like proteins have been identified so far.

Together with the cell cycle engine, a new type of control has evolved in higher Eukaryotes. Infact the cell proliferation machinery has to respond to all the environmental, extracellular stimuli that are present in a complex pluricellular organism. These controls have to be transduced at the level of cell cycle elements activating or inhibiting key transitional steps.

This new superimposed organization promotes the establishment of four major cellular states: growing, arrested, apoptosis and differentiation. Each of these states is defined by peculiar molecular features and is influenced by intra- and extra-cellular conditions. The equilibrium between the growing and the arrested states depends on the tissue organization and the availability of growth factors that are able to activate or inhibit key regulatory molecules involved in the control of the switch between these two states. These genes are often targets of mutation in tumoral cells.

During growth induction starting from a growth arrested state, two main gene expression programs must be controlled: the growth arrest specific pathway must be suppressed and the serum response pathway must be induced.

In this work I have analyzed some aspects of the control of cell proliferation.

At first I focussed my attention on the relationship between different conditions of quiescence. The operative

definition of a resting state has suggested that there are some differences in the final arrested state in relation to the method by which it is induced in cell culture. I cloned one gene induced only in serum starved cells and I have characterized it studying its changes in expression due to the presence/absence of different growth factors in different growth conditions. Then I analyzed its protein product finding that the pattern of expression at the protein level was very complex. This protein resulted to be cytoskeleton-associated.

After having defined a model to study the effects of different genes on the entry in S phase, I tried to dissect the effects of the major proliferation controlling molecule, c-myc, and to clone other genes that are able to interphere with this process. Focussing on c-myc, I analyzed the possible interaction with the apoptotic pathway that has been recently defined as one of the possible alternatives in the cellular proliferation fate.

Then I approached the cloning of genes that present a positive regulatory role on G1 progression. Work is now in progress on the characterization of these genes.

1. Serum Deprivation Response.

Two ways are commonly used for establishing growth arrest *in vitro*: growth factor deprivation and contact inhibition (2). It is still an open question whether these two growth arrest states are achieved and/or maintained through different mechanisms that rely on different gene expression programs (47) (78) (158) (165). I decided to approach this question by cloning

genes that are differentially induced by serum deprivation but not by contact inhibition in NIH3T3 cells.

I isolated one gene that is highly expressed at the mRNA level only after serum starvation: this gene was named *sdr* (serum deprivation response).

The induction of *sdr* mRNA seems to be sensitive to very low levels of growth factors (Fig. 1). This gene is infact immediately induced by 0.5% FCS but down-regulated in serum free medium. The transient *sdr* down-regulation at 24 hours after addition of 0.5% FCS could thus probably reflect the gradual consumption of available growth factors. However after 48 hours from the addition of 0.5% FCS or serum free medium *sdr* mRNA is induced at a similar level. This induction could be accounted for by either a common default programme to serum deprivation or by an autocrine *sdr* inducing loop which is activated only after cells have experienced the complete absence of serum factors.

A similarly complex pattern of *sdr* expression is found during Go->S transition induced by serum or by single growth factors ( ). When serum, PDGF and insulin are added to 0.5% FCS arrested NIH3T3 cells, *sdr* is clearly down-regulated after 6 hours but it appears to be transiently and highly reinduced after 12 hours (Fig. 2 and 3) ( ).

A different response is apparent when FGF and TPA are used (Fig. 4). They are able to down-regulate *sdr* at 6 hours but its reinduction seems to be stable and not transient.

The most striking difference in growth factor responsiveness is evident in the complete repression of *sdr* mRNA expression that is achieved during EGF treatment (Fig. 5) (76).

The oscillating expression of *sdr* gene is uncommon even if a similar pattern has been found in the immediate early gene CEF10 after the shift to the permissive temperature of a ts v-src mutant in Chicken embryo fibroblasts. Surprisingly this gene appears to have the same kinetics of regulation, albeit at different times, when using TPA (152).

Distinct subsets of 'immediate early' or 'secondary response' genes induced by different growth factors can probably account for the variable kinetics of *sdr* mRNA regulation within the 'in cycle' states elicited by the various stimuli (28,33-35). However the *sdr* mRNA pattern of expression seems not to be directly related to the entry in S phase since both insulin and TPA, although not efficient mitogens in NIH3T3 cells, affect *sdr* gene expression as serum, PDGF or FGF.

The complex pattern of *sdr* expression has attracted my attention into the mechanisms involved in its regulation. Nuclear run-on experiments have excluded a transcriptional regulation (Fig. 6), while analysis of *sdr* mRNA stability has pointed to a complex posttranscriptional control (Fig. 7). *sdr* mRNA half-life seems to be quite long when it is maximally induced after 48 hours of serum deprivation (at least 4 hours). The CHX block of the decrease of *sdr* mRNA stability during serum and EGF growth induction suggests an active role of the 'immediate early' genes in the regulation of its expression. However the effects of Act D addition during Go->G1 transition are more complex. Infact while the *sdr* serum response requires new RNA synthesis, EGF seems to activate an Act D insensitive pathway that is able to partially decrease *sdr* mRNA stability.

Moreover while the complex *sdr* serum response requires continuous protein synthesis both for the right titration of 'in cycle' *sdr* induced mRNA level and for its subsequent new down-regulation (Fig. 7), EGF needs active protein synthesis for *sdr* repression. This confirms that the 'secondary response' genes are probably involved in *sdr* regulation (75) even if I cannot exclude the presence of a feedback effect of *sdr* protein itself on its own mRNA. The complete repression of *sdr* expression after addition of EGF and the different effects with the use of transcription/translation inhibitors during EGF and serum growth induction suggest the existence of distinct largely non overlapping pathways between the cellular responses to serum and EGF addition (98).

Accordingly it has also been reported that antisense oligodeoxynucleotides against casein kinase II can inhibit the EGF but not the serum induction of DNA replication in human fibroblasts (127).

Furthermore a gene expression pattern specifically related to the EGF mitogenic stimulus has been recently elucidated through the study of genes that are induced in quiescent rat fibroblasts by reactivation of ts v-*src* and v-*fps* mutants (80).

The very complex effects of mitogens on *sdr* gene expression are probably due to the strict growth factor dependency of this gene at the mRNA level. When one compares the entry into quiescence induced by growth factor deprivation or by contact inhibition in terms of *sdr* mRNA it is possible to hypothesize the existence of a two step response. Initially repression of *sdr* mRNA is probably the result of a common general reset of cellular gene expression during growing->resting transition.

Later, when the quiescent state is fully established through the expression of common growth arrest specific genes, the *sdr* mRNA level is induced in the serum deprived but not in the contact inhibited cells. This difference is probably related to the different conditional 'restrictions': in the former the absence of cell to cell communication and the presence of low amounts of growth factors induce the expression of genes that will be more sensitive to the presence/absence of different mitogens; in the latter, the cell to cell and the cell/extracellular matrix interactions/communications are the most relevant restrictions even in the presence of a high level of growth factors.

I studied *sdr* mRNA regulation in other systems. By cloning the human cDNA and showing that the expression of *hsdr* mRNA in different growth conditions in IMR90 human fibroblasts resembles what was have already established for the murine counterpart. This result allows to hypothesize that *sdr* mRNA induction in serum starved cells is an element common to different cell lines suggesting its importance in the general cellular adaptation to these environmental conditions. Moreover the transient reinduction during G0/S transition was also confirmed.

The cloning of the human counterpart has allowed other analysis. The sequence of the human cDNA defined the *sdr* as a highly evolutionarily conserved gene. Infact the human and murine *sdr* deduced protein sequences have 82% homology. Moreover using two completely different approaches, a linkage map in mouse and *in situ hybridization* in humans, the results precisely mapped SDR in the proximal part of the murine chromosome 1, which is perfectly conserved in the synthenic region of the human

chromosome 2, where *hsdr* maps at 2q33. This region has been identified as a fragile site: being the relationship between these sites and known pathologies under investigation, a possible link of SDR to some inherited or molecular based diseases cannot be excluded (161).

The analysis of the Data banks searching for homologies gave two interesting results. Infact three microsequenced peptidic fragments of a protein purified from platelets are almost perfectly conserved in sdr protein sequence. The protein is named PS-p68 and it has been studied for its ability to bind phosphatidyl-serine, a common molecular component of cellular membranes. Furthermore the N-terminus portion of a fused oncogenic protein 7N-1 has resulted to be highly similar (59.5%) to the sdr deduced aa sequence. This protein is constituted at the N-terminus by a still unknown protein fused to a truncated form of the cellular proto-oncogene c-raf (96) (156) (164). This Serine-Threonine protein kinase presents a regulatory domain on its N-terminus and commonly most of its activated forms lack this region by substituting it with different cellular proteins. The role of this sdr/like sequence is not strictly related to the activation of c-raf but probably reflects either a passive inhibition of a repression or an active increase in the half-life. However this homology is significant because it suggests that sdr is probably a member of a protein family.

By expressing SDR in bacteria, the fusion protein recovered was used to produce policlonal antibodies. The pattern of SDR protein distribution under different cell growth conditions is complex. The neo-synthesized level of sdr protein reflects the pattern already characterized for its mRNA. However while in

growing cells SDR appears as a doublet, in both growth arrested cells only the slower migrating form is present. These two bands are postranslational modifications of SDR protein the smaller size representing the phosphorylated form. The total amount of SDR protein under different growth conditions was also analyzed by immunoblotting. The result was quite surprising. While the total level of SDR protein in serum starved human fibroblasts is induced with respect to growing cells, density dependent inhibited cells present a large increase in SDR protein content compared to the other growth states. Thus SDR protein level seems completely unrelated to SDR mRNA. This is probably due to a continuous accumulation of the protein during the establishment of the culture of high cell density. However SDR remains to be differently expressed in these two growth conditions: the protein is infact highly induced in contact inhibited cells. During G0/G1 transition the level of SDR protein sligthly decreases after 4 hours from serum addition but then is reinduced. However SDR protein level is completely shut off after 24 from growth induction.

Next I focussed my attention on the intracellular distribution of SDR protein. In serum starved cells there is a patchy uniform cytoplasmic-distribution with some spots. Interestingly in some cells there is a regular distribution localized on the lower side of the membrane and at the edge of the fibroblasts where it sometimes appears to organize into linear arrays.

Using the PMG2 buffer as a pre-extraction step during immunofluorescence analysis, I was able to confirm that a large amount of the cellular SDR protein is tighly linked to the

cytoskeleton compartment. This result was also confirmed in transfection experiments of COS-7 cells.

The results obtained so far do not perfectly match those obtained by R. Burgener et al. in their characterization of the purified PS-p68. Some features of the proteins are indeed in common: sequence homology, mobility in polyacrilamide gel electrophoresis, presence of a doublet form, type of postranslational modification and partial submembrane localization in immunofluorescence analysis. However some differences do exist: both forms of PS-p68 are phosphorylated while SDR presents only one phosphorylated band; PS-p68 does not appear to be cytoskeleton-associated while a large portion of SDR protein is present in the insoluble form after treatment with PMG2 buffer.

I can hypothesize two possible models to explain these data. One model defines PS-p68 and SDR as same protein: the different phosphorylation state can be due either to different spliced forms of PS-p68/SDR or to the presence of different cellular proteins that interact with PS-p68/SDR in different cell types. This hypothesis can also explain why PS-p68 is not cytoskeleton-associated in platelets. According to the second model, PS-p68 and SDR are two different proteins of two highly related genes.

I feel that the first model fits better even if a final answer to the question of the relationship between these two proteins requires other *ad hoc* experiments.

The phosphatidylserine-binding proteins were previously described to bind both phosphatidylserine and protein kinase C and this binding was inhibited after phosphorylation. They were considered as possible regulators of the compartimentalization

of PKC, which is known to be translocated from cytosol to the plasmamembrane during activation. It was also postulated that these proteins can have anchoring functions for the association of PKC with the cytoskeleton.

PS-p68 has peculiar aspects among this class of proteins. It does not bind Ca^{2+} and does not require Ca^{2+} for its binding of phosphatidylserine, in contrast with most known phospholipid-binding proteins. In blood cells PS-p68 is present only in platelets. Very interestingly the activation with thrombin induces a slight decrease in the total amount of PS-p68 while activation with TPA and carbacyclin does not. This protein resulted to be a substrate for protein kinase C *in vitro* even if its level of phosphorylation does not change after cellular treatment with TPA.

Moreover the homology with the cellular protein fused to c-raf can suggest the existence of a SDR/related multigene family.

I then tried to better understand the relationship between SDR and the cytoskeleton network. The double immunofluorescence analysis shows a partial overlapping staining with microtubules and, to a minor extent, with vimentine. When the distribution of these two networks was modified by adding drugs that are able to solubilize and form crystal structures of tubulin, the SDR distribution was highly affected as seen by immunofluorescence analysis. When a parallel biochemical analysis was performed it indicated that while tubulin solubility is dramatically affected, the nocodazole and the taxol additions have no effects on SDR suggesting that this protein is not directly associated to tubulin.

In order to assess if SDR can exert a growth regulatory role, the human cDNA has been cloned under an SV40 promoter and microinjected in resting cells. After microinjection, cells were induced to enter into cell cycle by 20% FCS addition in the presence of BudR. After 18 hours cells were fixed and a double immunofluorescence analysis was performed. The ectopic expression of SDR had no effects on the S phase entry (data not shown). Other experiments have been performed in order to see if SDR is able to modify the cytoskeleton organization but no effects were observed.

The SDR protein studies have confirmed that differences in gene expression between the two quiescent states do exist. Even if in a opposite way, SDR protein appears to be highly accumulated in density dependent inhibited cells. Moreover the pattern of phosphorylation in growing and resting cells is different suggesting that this protein is indeed under a regulatory control during different cell growth conditions.

2. G1 progression control: c-myc.

Among all the 'immediate early' response genes, c-myc has a special role: it is infact expressed not only during G0/G1 transition but also in actively growing cells. Moreover when environmental restrictions imposed to the cell the entry into quiescence, c-myc is immediately repressed. The ectopic expression of c-myc in growth arrested cells provokes a very complex effect: infact together with its involvement in proliferation control, c-myc is able to activate the cell death program. These two effects are intimately associated and

probably reflect the two possible cellular responses to the presence of the c-myc protein: apoptosis and proliferation. The switch between these two cellular states is determined by the presence/absence of growth and survival factors in the medium.

My purpose was to define the growth inducing capacity of c-myc by inhibiting the induction of apoptosis. Programmed cell death was inhibited in two ways: after microinjection of c-myc in serum starved cells, these were treated either with PMA (that exerts a survival potential), or with 2% FCS that not only suppresses apoptosis but also induces the re-entry into the cell cycle in part of the cell population. In both conditions, ectopic expression of c-myc was able to activate entry in S phase.

Since this activatory property was not due to a shorter G0/S transition (82); I can deduce that the kinetic of S entry in the microinjected cells is the same as for 20% FCS activated cells; that is the presence of c-myc is able to increase the number of cells that are able to respond to these mitogenic stimuli.

The analysis of c-myc mutants in both systems resulted particularly interesting. Deletion of the region responsible for the heterodimeric formation with Max was absolutely required for c-myc mitogenic effects. This result confirms the central role of this domain since all the biological effects of c-myc, as analyzed *in vitro* and *in vivo*, have always been inhibited by deletion of its C-terminus. The dissection of the N-terminus region, where both the transactivation activity and the ability to bind *in vitro* the Rb protein reside, is more complex. At first I have shown that the mutant Δ106-143 is completely unable to activate entry into S phase. This result is not surprising since

this region is absolutely required for all known c-myc activities. The region containing the first 100 aa was analyzed using three mutants: Δ7-91, Δ41-53 and Δ56-103. The larger deletion completely abolishes the REC cotransforming activity, the Rat-1a cell transformation and the induction of apoptosis. Δ7-91 mutant is infact completely unable to activate the entry in S phase. On the contrary Δ56-107 has a mitogenic effect equivalent to wt c-myc while it has been reported that this mutant has only 10% of the wt REC cotransforming ability. The Δ41-53 mutant is able to activate S phase entry with a 60% wt capacity as for the REC cotransforming assay. Another interesting result comes from the analysis of Δ265-317 mutant. This deletion provokes different effects in different assays: it affects severely the transformation ability while it has 50% wt capacity in the cotransformation assay. Moreover it is dispensable for the induction of apoptosis and the transformation of chicken fibroblasts but is absolutely required for macrophage transformation. This mutant is able to activate the entry in S phase at 50% relative to the wt. This property is similar to the cotransformation assay. It has been proposed that these differences in different assays are probably due to particular interactions that might be necessary only in certain conditions. Infact none of the three active mutants in the 2% FCS system are able to activate the entry in S phase in the PMA treated cells.

3. G1 progression control: the molecular cloning of positive regulatory genes.

The existence of growth activatory and inhibitory mRNAs is very well known. In different systems, such as T lymphocytes and hepatocytes, specific mRNAs have different effects on the entry in S phase of recipient cells dependeng on the growth conditions of the donor cells. Infact while mRNAs extracted from normal liver cells contain growth inhibitory molecules, mRNAs extracted from regenerating liver do not. The same analysis has been previously performed in this laboratory where the presence of growth inhibitory mRNAs was demonstrated in arrested NIH3T3 cells. On the contrary growing NIH3T3 cells contain mRNAs that are able to activate the entry into S phase. (M. E. Ruaro; manuscript in preparation).

Using the same system as for c-myc analysis, I tried to clone the gene responsible for the observed growth activatory effect. I approached this problem in three steps:

1. identification of the fraction of size selected poly(A)+ containing the mRNA responsible for this effect and its subsequent cloning

2. preparation of a 'growing' specific probe to screen the previously described library

3. functional assay for each clone that is 'growing' specific and of the expected size.

The results of the microinjection of the sucrose gradient sedimentation fractions of growing poly(A)+ have shown that the growth activatory property is associated mainly to only one fraction containing mRNAs of 550 nt. The same experiment was performed using the 'arrested' poly(A)+ where no activatory effects were observed in the fraction of the same size indicating that the growth inducing gene is specifically expressed in

growing cells. No activatory effects were observed also in the c-myc mRNA containing fraction. Different explanations could be suggested: the presence of a growth inhibitory mRNA in the same fraction, the low abundance of the c-myc mRNA in growing cells, the small half-life of the mRNA. Finally I orientally cloned the sized mRNA in a lambda vector.

At this point I synthesized a substracted probe to screen the cDNA library for growing specific clones. A combined use of PCR and SA-PMP techniques allowed me to perform a very effecient subtraction protocol and to obtain growing specific cDNAs. I used these cDNAs to screen the oriented library and to isolate several genes. Up to now I have identified two clones that were already known: P198 and L29.

In vitro mutagen treatment of tumor cell lines generates immunogenic variants that are rejected by singenic mice (43) (101) (151) (163). Since they fail to form tumors, these variants have been named *tum*$^-$, as opposed to the original *tum*$^+$ cells. P198 is the gene responsible for the *tum*$^-$ variant obtained after mutagenic treatment of P1, a clonal cell line derived from mouse mastocytoma P815. Many tum$^-$ variant reponsible genes have been cloned so far: they are completely unrelated and are localized in all the subcellular compartments. The P198 biochemical and functional characteristics are still unknown.

L29 is a ribosomal protein (15) (88). The translational apparatus is a very important regulatory target during cell proliferation. The total translational rate is increased in actively growing cells in respect to quiescence. Regulation of translation occurs primarily at the initiation step when the mRNA binds to the ribosome. The trans-acting factors that function in mRNA

binding include at least three initiation factors: eIF-4A, eIF-4B and eIF-4F. eIF-4F itself is composed of three polypeptides. Its smallest subunit, named eIF-4E, when overexpressed, is able to transform NIH3T3 cells. Moreover it is able to induce the entry in S phase when microinjected into quiescent fibroblasts. It has been recently shown that eIF-4E is able to exert its mitogenic activity increasing the amount of activated $p21^{ras}$.

eIF-4E, like the ribosomal protein S6, becomes phosphorylated after addition of serum and single growth factor to quiescent NIH3T3 cells.

I still have to analyze if these two clones are responsible for the S phase inducing effect. If not I have to purified other plaques that were positive during the primary screening.

MATERIALS and METHODS

1. Cell lines and cell culture conditions.

NIH3T3 cells (kindly provided by Dr. R. Muller) and IMR90 human diploid fibroblasts were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), penicillin and streptomycin (100 U/μg/ml). IMR90 cells were used within 10 and 20 passages for all the experiments.

For serum starvation, the medium was changed to 0.5% FCS when cells were subconfluent: cells were then left in the same medium for 48 hours (NIH3T3) or 72 hours (IMR90). Under these conditions, labelling with 50 μM BrdU for an additional 24 hours resulted in labeling of less than 5% of the nuclei.

For DNA synthesis induction, fresh medium containing 2%, 10% or 20% FCS was added to the arrested cells. Cells were then harvested at the desired times for RNA isolation and other analysis. After 24 hours of BrdU incorporation, more than 90% of the nuclei scored positive.

For density dependent inhibition, cells were plated at $10^4/cm^2$ in 10% FCS. 24 hours after plating (considered as starting point for growing cells) the medium was changed every 2 days. After 4 days in culture, less than 5% of the cells are able to incorporate BrdU when incubated for two hours. Exponentially growing cells are cultured for 24 hrs in 10% FCS. After this time incubation with BrdU for 2 hrs resulted in 40 % incorporation.

v-myc transformed NIH3T3 cells (a kind gift of Dr. F. Tato', Uniersity of Roma) and COS-7 cells were cultured in the same conditions as above.

In some experiments, the entry into DNA synthesis was induced by addition of single growth factor at the concentration of, 20 ng/ml for PDGF (kindly provided by C. H. Heldin, Ludwig Institute, Uppsala), 100 ng/ml for bFGF (kindly provided by C. Grassi, FarmItalia, Milano), 40 ng/ml for EGF (kindtly provided by A. Ulrich, Max Planck Institute, Munich), 100 µg/ml for insulin (porcine insulin, Sigma Chemicals), 10^{-7} M TPA (Sigma Chemicals).

When used, Cycloheximide and Actinomycin D concentrations were, respectively, 10 and 5 µg/ml. Cycloheximide was able to inhibit more than 95% of protein synthesis as determined by [^{35}S] methionine incorporation and TCA precipitation.

2. DNA synthesis assay.

Cells grown on cover slips (20 by 20 mm) in the same culture dishes from which RNA or protein extracts were prepared were incubated for one hour in the presence of 50 µM BrdU (Boehringer). After this time they were fixed for 20 min in 3% paraformaldehyde and permeabilized with 0.1% Triton X-100. DNA was then denatured by treatment for 10" with 50 mM NaOH. After neutralization with PBS the coverslips were incubated with mouse monoclonal antibody against BrdU (Amersham) for one hour at 37° C (64). The second antibody was TRITC conjugated rabbit anti-mouse immunoglobulin antibodies (Southern Biotechnology). Total nuclei were visualized with Hoechst 33342 (1 µg/ml). The

percentage of activation was calculated as the ratio between cells positive for TRITC and total cells.

3. Purification of genomic DNAs.

The genomic DNAs were extracted from cell lines according to S. Gustincich et al. (67).

4. Total RNA and poly(A)+ preparation.

Total RNA was extracted from cultured cells or animal tissues as previously described (29). The murine organs were conserved at -80° C. The poly(A)+ fraction of total RNA was prepared using an oligo(dT) cellulose (Stratagene, San Diego).

5. Construction and screening of cDNAs and genomic libraries.

cDNA libraries were constructed using 4 μg of poly(A)+ mRNA extracted from quiescent cells starved for 48 hours in 0.5% FCS, human liver mRNAs and size selected poly(A)+ mRNAs from growing cells.

First strand cDNA synthesis was performed using an oligo (dT) or random primers as previously described. cDNA was ligated to EcoRI/HindIII linkers and cloned, when possible, in an oriented way (46) into the lambda vector λGDST3/T7 (66) (68). Phages were packaged using the Gold Packaging System (Stratagene) and plates in JM101 E. coli cells.

For screening of the serum starved library, 5 x 10^4 plaques of the amplified library were plated at a density of 5000 PFU/150-mm petri dish. Three lifts were made from each of ten petri dishes using nylon membranes (Gene Screen). The first lift from each plate was probed with a hot single strand cDNA from serum starved cells; the second lift was probed with cDNA from contact inhibited cells and the third one with cDNA from cells harvested after 6 hours from the addition of 20% FCS to growth arrested cells. Plaques that specifically hybridized only with the cDNA probe from serum starved cells were isolated and rescreened.

For screening of the human cDNA library, an aliquot was plated at a density of 40000 PFU/150-mm petri dish. Four petri dishes were lifted into nylon membranes (Gene Screen) and probed with a 1.7 Kb fragment of the murine *sdr* cDNA containing the whole ORF. The hybridization was performed at low stringency condition (SSPE 6x, SDS 1%, herring sperm DNA 100 mg/ml at 50° C) and the washes in Sodium Phosphates salts 0.5 M, SDS 1% at 40° C. Plaques that hybridized with the cDNA probe were isolated and rescreened.

A human genomic library cloned in λEMBL3A was kindly provided by Prof. C. Santoro (University of Trieste, Italy). For screening, an aliquot of the library was plated at a density of 40000 PFU/150-mm petri dish. 25 petri dishes were lifted into nylon membranes (Gene Screen) and probed with a 300 bp fragment of human *sdr* cDNA. The hybridization was performed at high stringency condition (NaCl 1 M, SDS 1%, herring sperm DNA 100 mg/ml at 68° C) and the washes in SSC 0.2x, SDS 1% at 68°

C. Plaques that hybridized with the cDNA probe were isolated and rescreened.

6. Northern blot analysis.

Total cellular RNA (10 µg) was fractionated on 1% agarose gel containing 6.7% formaldehyde. Integrity and relative amount of RNA were analyzed by ethidium bromide staining. Gels were transferred to a Duralon-UV nylon membrane (Stratagene). RNA was cross-linked by exposure to UV light (Stratalinker, Stratagene). Probe was prepared by random primer oligolabeling and hybridization was performed in 1 M NaCl, 1% SDS at 65° C using $5x10^{5}$ c.p.m. per ml. Washes were performed as follows: 2x SSC room temperature twice 5'; 2x SSC, 0.1% SDS 65° C twice 15'.

7. DNA sequencing.

DNA fragments were sequenced in λGDST3/T7 or in pGDSV7 vectors as previously described (44) using the T7 DNA Polymerase (Pharmacia). Synthetic oligonucleotides were also used as primers for the sequencing reactions. The entire sequence were read on both strands.

8. Run-on experiment.

For the isolation of nuclei, cells were washed twice with ice cold PBS and removed with a rubber-policeman. The pellet of cells was incubated for 5' in lysis buffer (10 mM Tris-HCl pH 7.4,

10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP40). The lysate was centrifuged at 500x g and the pellet resuspended in the storage buffer (10 mM Tris-HCl pH 8.3, 40% glycerol, 5mM $MgCl_2$, 0.1 mM EDTA pH 8) and left in liquid N_2 until use.

Run-on transcription assay was performed as previously described (65). The DNA was spotted onto a nylon membrane (Stratagene); 10 µg of previously denatured DNA (0.25 M NaOH for 20' at room temperature, neutralized by addition of an equal volume of 0.25x SSC) was applied per slot. Hybridization was performed in 1 M NaCl, 1% SDS, 1 mg/ml heparin, 100 µg/ml salmon sperm DNA at 65° C for 36 hours with a probe concentration of $2x10^6$ c.p.m./ml. Filters were then washed with 2x SSC at room temperature twice for 10'; with 2x SSC, 1% SDS at 65° C for 15', and finally with 0.2x SSC, 0.1% SDS at room temperature for 10'.

9. Protein expression in *E. coli.*

Sdr was expressed in E. coli as a Glutatione transferase (GST) fusion protein. A 1.2 Kb (112 a.a.- 425 a.a.) PCR fragment of the murine cDNA was created using two oligos containing BamHI and EcoRI sites and cloned in frame in the polilinker of the pGEX-3X vector (Pharmacia). Recombinants were used to trasform competent DIH-101 E. coli cells and to express the fusion protein.

The bacterial extract was incubated with Sepharose-GST beads (Pharmacia) for one hour at 4° C in a rotating platform. The beads were washed 3 times with PBS 1% Triton X-100 and eluted by two incubations with one bead volume of freshly prepared elution buffer (15 mM glutatione, 50 mM Tris-HCl pH 8). The

eluted proteins were separated by gel-electrophoresis and stained with Coomassie blue in water (0.05%). The band of expected size was recovered and electroeluted.

10. Affinity purification of rabbit antisera.

Rabbits were immunized with 100 µg of electroeluted protein, diluted 1:1 in complete Freund 's adjuvant (Sigma). The same amount of fusion protein was introduced into rabbit in incomplete Freund's adjuvant at 15 days intervals for 2 months.

The rabbit antisera was purified by cromatography on an affinity column. 5 ml of immune sera diluted with an equal volume of PBS were precleared on a cromatography column containing total protein extract from noninduced E. coli crosslinked to an Affi-prep 10 (Bio-Rad) support. The non bound fraction was loaded on a Glutatione transferase Affi-prep 10 column and subsequently on the affinity Sdr Affi-prep 10 column.

For the affinity purification of the antibodies 400 µg of electroeluted bacterial protein were coupled to the Affi-prep matrix.

The column was washed with 50 bead volumes of PBS, then eluted with 1 ml of 0.2 M Glycine HCl pH 2.8 and the fractions neutralized with 1 M Tris pH 8.

11. PCR

The PCR reactions were performed using the Perkin Elmer-Cetus Corporation anplification machine (141) (60). The following reagents were mixed in a test tube: 10 µl of PCR buffer 10x (25

mM $MgCl_2$, 0.5 M KCl, 0.1 M Tris HCl pH 8.4), 200 μM dNTPs, 50/200 μM primers and the template. After boiling 5', 0.5 μl of AmpliTaq DNA Polymerase (Perkin Elmer) were added. The PCR cycles are variable according to the different needs. Each cycle has three main steps: denaturation, annealing and elongation.

12. *In Vitro* transcription/translation.

For in vitro transcription, λGDST3/T7 was digested at the 3' end of *hsdr* and transcribed with T7 polymerase (Promega) in presence of 7mGpppG CAP (Pharmacia).

The capped mRNA, 1 μg, were translated in rabbit reticulocyte lysates (Novagene) supplemented with ^{35}S methionine (ICN Tran^{35}S label).

5 μl of the translation product was diluted with 100 μl of 150 mM NaCl, 50 mM Tris pH 8, 0.1% NP40 and immunoprecipitated for 30' on ice. After this time 50 μl of Sepharose protein A (Pharmacia) (10% w/v) were added to recover the anti *Sdr* antibody, and the incubation prolonged for 30' at 4° C on a rotating platform.

After 3 washes in the same buffer the complex was resolved on SDS polyacrylamide gel, which was fluorographed, dried, and exposed to film.

13. Immunoblotting.

For Western immunoblotting, total cellular lysates were prepared adding Laemli sample buffer (2% SDS, 100 mM DTT, 60 mM Tris ph 6.8) to the cell pellet previously washed in PBS.

The cell lysates were checked for equal amount of proteins on parallel gel stained by Coomassie blue.

Proteins were transferred to a 0.2 μm pore size nitrocellulose membrane (Schleicher & Schuell) using a semidry blotting apparatus (Biorad). Transfer buffer was 20% methanol, 48 mM Tris, 39 mM glycine, 0.04% SDS.

Filters containing transferred samples were stained with 0.1% Poinceau S in 5% acetic acid (Sigma) to identify the position of molecular weight standard. Non specific protein binding sites were blocked by two hours incubation in Blotto-Tween 20 (50 mM Tris pH 7.5, 200 mM NaCl, 5% nonfat dry milk and 0.01% Tween 20) at room temperature.

For immunological detection of proteins, primary antibody was then added in Blotto-Tween 20 and allowed to react overnight at room temperature.

Unbound primary antibody was removed and the filters were rinsed three times with Blotto-Tween 20. Horse radish peroxidase labeled second antiboby (Southern Biotechnology) was added and incubated for one hour at room temperature, followed by three washes to remove unbound antibody.

After an additional wash in PBS the membrane was immunodetected with enhanced chemiluminescence (ECL) (Amersham), and exposed to Kodak film for an appropriate time.

14. Preparation of cell extracts containing soluble and cytoskeletal fractions.

To prepare soluble and cytoskeletal fractions of serum deprived fibroblasts, cells grown on a 10 cm dish were washed

twice with PBS (at 37° C), and then two times with microtubule stabilizing buffer (0.1 M Pipes pH 6.9, 5 mM EGTA, 4% polyethylene glycol 8000).

Soluble proteins were extracted by lysis in 1.5 ml of stabilizing buffer containing 0.5% Triton X-100 and proteases inhibitors (1 mM phenylmethylsulphonyl fluoride and 20 µg/ml leupeptin, pepstatin, antipain, aprotinin) for 5 min at 37° C.

The solution was removed and an equal volume of 2x Laemli buffer was added to the soluble fraction. The remaining cytoskeletal fraction was solubilized in Laemli gel sample buffer and DNA was removed after boiling by centryfugation.

After addition of tRNA carrier to 100 µg/ml, both fractions were concentrated by precipitation with 10 vol of cold methanol.

After 12 hours at -20° C, precipitated proteins were recovered by centrifugation and the dried pellet resuspended in appropriate volumes of Laemli gel sample buffer.

Samples were boiled and checked for equal amount of total proteins on separated gel before performing the SDS PAGE for the blotting.

15. Immunoprecipitation.

IMR90 fibroblasts were metabolically labeled with 1 mCi/ml ^{35}S methionine (ICN Tran^{35}Slabel,1026 Ci/mmole; 1mCi= 37 MBq) for the appropriate time in 1 ml of DMEM methionine free medium (Sigma).

Radioactive labeled cells were lysed in 0.5 ml of SDS lysis buffer (0.8% SDS, 100 mM NaCl, 50 mM Tris pH 7.5) and boiled for 3'. Then the lysates were diluted with 0.5 ml buffer B (4% Triton

X-100, 100 mM NaCl, 50 mM Tris pH 7.5) and additionated with 40 µl of nornal rabbit serum. One hour later the labeled extracts were used to resuspend 30 µl wet volume pellet of *Staphilococcus Aureus*. The lysates were left on a rotating platform for one hour at 4° C, and then briefly centrifuged in an Eppendorf centrifuge. This step was repeated one more time and the resulting supernatants used to immunoprecipitate.

The lysates were incubated with the Sdr antibody for 2-4 hours at 4° C with continuous rocking, then they were added to 100 µl of protein A Sepharose (Pharmacia) (10% w/v) and further incubated for one hour.

After three washes with buffer B, the resin was resuspended in Laemli sample buffer and the complexes released by boiling for 5'.

For the ^{32}P labeling the cells were grown in DMEM 0.5% FCS for 72 hours before lysis; during last 12 hours the DMEM was changed with a phosphate free medium supplemented with 0.4 mCi/ml ^{32}Pi.

16. Phosphatase digestion.

Protein were immunoprecipitated from asinchronous growing fibroblast culture as described above, except that samples were washed twice with phosphatase reaction buffer (50 mM Tris HCl pH 8, 10 mM $MgCl_2$, 150 mM NaCl), supplemented with 0.1% Triton X-100, 0.05% SDS, 2 mM phenylmethylsulfonyl fluoride (PMSF). After 3' at 68° C, samples were divided in half and incubated for 3 hours at 37° C, with or without 3 U of molecular biology grade bacterial alkalyne phosphatase.

(Pharmacia). The reaction was terminated by addition of Laemli sample buffer.

17. Immunofluorescence microscopy.

IMR90 cells were grown on glass coverslips as described previously. Cells were fixed for 10' each in methanol followed by acetone at -20° C, in some cases after preextraction in 100 mM Pipes pH 6.9, 5 mM $MgCl_2$, 2mM EGTA pH 8, 0.1% Triton X-100 2 M Glycerol for 2' at room temperature. To perform double immunofluorescence, fixed cells were labeled with affinity purified *Sdr* and mouse monoclonal in PBS 3% BSA for one hour at 37° C. Coverslips were then rinsed twice in PBS and further incubated with a goat anti rabbit biotinilated antibody (DAKO).

The immunocomplexes were visualized by incubation with a Texas red labeled goat anti mouse antibody (Southern Biotechnology) and streptavidine-FITC (Jackson) in PBS.

A Zeiss microscope was used for all the experiments with the appropriate set of filters for fluorescence.

18. Transfection.

COS-7 cells were grown on coverslips in 35 mm petri dishes containing 5 x 10^4 cells per dish seeded in DMEM containing 10% FCS. After 24 hours, cells were washed twice in serum free medium. 400 µl of the transfection mixture (360 µl serum free DMEM, 1.3 µg DNA, 40 µl 5 mg/ml DEAE/dextran) were added and left for 30' at 37° C in a 5% CO_2 humidified atmosphere. Then the medium was changed with 2 ml of 10% FCS

containing 100 μM cloroquine and left for 3 hours. After medium removal, cells were cultured for additional 24 hours in 10% FCS before performing immunofluorescence analysis.

19. Microinjection experiments.

For microinjection assays, cells were grown on coverslips in 35 mm petri dishes containing 10^5 cells per dish seeded in DMEM containing 10% FCS. After a 24 hours incubation at 37° C in a 5% CO_2 atmosphere, the culture medium was changed to DMEM containing 0.5% FCS and incubated for additional 48 hours to obtain resting cells.

Microinjection was performed using the Automated Injection System (Zeiss, Oberkochen, Germany). Cells were injected with 50 ng/μl expression vector of cesium chloride ultracentrifugation purified DNA. Each cell was injected for 0.5 s at the constant pressure of 150 hPA. Under these conditions approximately 0.05 pl of sample was injected, corresponding to about 500 copies of the plasmid. After injection, cells were incubated at 37° C in a 5% CO_2 atmosphere. DNA synthesis assays were performed as described above. In some experiments, the medium was changed and BrdU was introduced for the indicated times. c-myc gene product was immunostained using an anti myc rabbit policlonal antibodies gently provided by Dr. R. Eisenmann (F. Hutchinson Institute, Seattle, Washington) and affinity purified by S. Goruppi followed by swine anti-rabbit FITC-conjugated antibodies (Dakopatt, Glostrup, Denmark). In the co-microinjection experiments, a solution containing the plasmid of interest and the hu-TR cDNA cloned in the same expression vector

has been used. The transferrin receptor was visualized by a human specific anti TR/OKT-9 monoclonal antibodies (IgG1-Ortho Diagnostic Systems, Raritan, NJ) followed by goat anti mouse IgG1 FITC-conjugated antibodies (Southern Biotechnology Associates, Inc., Birmingham, AL).

The poly(A)+ fraction of RNAs were microinjected at a concentration of 1 μg/μl in the presence of the fluorescent dye dextran.

20. Sucrose gradient sedimentation.

20 μg of the poly(A)+ fraction of total RNA was boiled for 1' in the presence of 0.1% of SDS and immediately loaded on the sucrose gradient. This is prepared starting from two sucrose solutions of 20% and 40% weigth/volume in a buffer containing 0.1 mM EDTA, 10 mM Tris HCl pH 7.6 and 0.1% SDS. After 12 hours of ultracentrifugation at 2° C at 40000 r.p.m., 24 aliquots of ~200 μl were collected from the bottom of the tube. Each aliquot was precipitated in the presence of glycogen and resuspended in 20 μl of water.

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