ISAS - International School for Advanced Studies

Molecular cloning and characterization of a gene specifically expressed in quiescence

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INTRODUCTION

1. Definition of quiescence:

Most cells in the body do not divide and are in quiescent state with unreplicated DNA. Although some cell lineages, such as neurons and muscle cells, appear to have lost the capacity to divide, others such as liver cells and lymphocytes are still able to enter the cycle under the influence of appropriate stimuli. For instance, liver cells will proliferate during the regenerative process while a lymphocyte upon stimulation with an appropriated antigen.

By alteration of their growth conditions, cells in culture can be brought into a state of quiescence such that proliferation ceases. This non proliferative state is termed Go and in turn cells can be induced by appropriate stimuli to return from Go back into the cell cycle.

Ways in which cultured cells can be induced into quiescence include deprivation of growth factors as a consequence of lowering the concentration of serum in growth medium, or allowing the cells to become overcrowded.

Proliferation can then be restored in the former case by replenishing the serum or in the latter by seeding the cells at a lower density.

Even if Go and G1 have the same content in chromosomal DNA, they differ in many molecular and biochemical properties. Go cells are smaller: protein and RNA molecules are not rapidly resynthesized after degradation, macromolecular synthesis is in general one third lower in Go as in proliferating cells. Enzymes and transmembrane transport activities are low in Go cells and ribosomes are monosomal rather than polysomal (1).

For many years it was an open question if quiescence is the basal house-keeping life of the cell that needs just positive stimuli in order to go into the cell cycle or if the growing and the resting state are the result of a very complicated network of regulatory signals that can induce both the "out of cycle" phase and the "in cycle" one. The consequence of the validity of this second model is that Go is a very well determined state of the cell with peculiar features and properties.

2. Detection of the existence of a negative growth control:

In order to answer to this very important question two approaches have resulted very useful: the analysis of

cellular hybrids and the microinjection of particular poly(A)$^+$ mRNAs.

The regulation of DNA synthesis in normal human cells has been analysed by fusing together cells in different replicative states. Cycling HDF has resulted to be unable to induce DNA synthesis in non cycling quiescent HDF cells; that is the cycling HDF were inhibited from entering S phase. These data suggest that quiescent HDF contain an inhibitor of entry into S phase. On the contrary young HDF were able to induce DNA synthesis in cycling HDF. Taken together, these two sets of data suggest that maintenance of normal HDF in a non replicative resting state involves a negative control whereas the timing of initiation of DNA synthesis in cycling cells involves a positive control mechanism (2).

Another approach consists in the microinjection of poly(A)$^+$ mRNAs expressed in resting or growing cells into acceptor cells to score for the presence of inhibiting or activating effect on the entry in S phase.

With this analysis it was pointed out that the presence of an mRNA in NIH3T3 cells after 24 hours of serum starvation is able to inhibit the entry in S phase of activated cells. It was also seen that RNA extracted from growing cells are able to activate the entry in S phase of resting cells.

RNA responsible for the inhibition of the entry in S phase was detected also in two other systems:

-naturally quiescent human lymphocytes, consisting predominantly of T cells, contain mRNAs that can inhibit DNA synthesis when injected into either human diploid fibroblast (IMR-90) or transformed recipient cells (HeLa) (3).

-normal rat liver cells mRNAs can inhibit DNA synthesis in human diploid fibroblasts (4).

From this data comes out the validity of the model concerning the existence of a balance between positive and negative growth controls and the necessity of understanding better the peculiar features of growth arrest (5).

3. Entry into quiescence:

The effect of serum starvation on the ability of cells to progress through the cell cycle is very rapid. Exposure to serum-free medium for 15-30' is sufficient to arrest cells in the cell cycle for several hours. Although protein synthesis is equally depressed in all cell cycle stages after a brief serum starvation, the cellular progression through the cycle is affected in a highly stage specific manner. Only postmitotic cells younger than 3-4 hours are inhibited in their progression through the rest of the cell cycle. Cells older than 4 hours, on the other hand, are able to trasverse the remaining part of the cell cycle with undiminished speed in the absence of serum, although protein synthesis is reduced by 50%. On the basis of the cell-cycle arrest induced by brief serum starvation, the G1

period of exponentially proliferating 3T3 cells can be subdivided into two physiologically different phases: one post-mitotic (G1pm) and one pre-DNA synthesis (G1ps). It's during G1pm that the cell senses the environment and can refuse to divide (6).

4. Negative growth factors:

As the positive branch of growth control present multiple factors that are able to activate the proliferation of a cell, during the last years examples of factors that are able to inhibit cell growth have been identified (7).

TGF-ß1 was first detected by its ability to cause phenotype transformation of rat fibroblast (8). Now it is clear it is involved in numerous regulatory actions in a wide variety of both normal and neoplastic cells (9) (10).

Particularly it was shown that it is essentially identical to a growth inhibitor that had been previously identified and partially characterized in monkey kidney cells and which inhibited the proliferation by an autocrin mechanism.

More recently, it has been shown that TGF-ß1 is a strong inhibitor of proliferation in many primary or secondary cells including hepatocytes, embryo fibroblast, T and B lynphocytes, keratinocytes and bronchial epithelial cells.

TGF-ß1 is able to inhibit the progression of serum-stimulated quiescent primary fibroblasts through the cell cycle as well as inhibit cell proliferation of actively growing cells.

Most established cells loose response to the antiproliferative effects of TGF-ß1 and actually in some cell lines TGF-ß1 stimulates growth.

The mechanism by which TGF-ß1 mediates growth inhibition following binding to its cell surface receptor is not completely understood. While EGF stimulated DNA synthesis is inhibited by TGF-ß1, there is no interference with EGF binding to its receptor or with several well-characterized early effects of this interaction. It has been demontrated that TGF-ß1 depresses c-myc epression through inibition of transcriptional initiation. It was hypothized that this reduction of c-myc by TGF-ß1 may play an integral role in TGF-ß1 mediated inhibition of keratinocyte growth, since continual expression of c-myc was found to be necessary for keratinocyte proliferation.

Recently it has been shown that TGF-ß1 and the protein product of the tumor suppressor Retinoblastoma gene may partecipate in a common growth-inhibitory pathway.

Infact TGF-ß1 prevents Rb phosphorylation in late G1 and this event can be sufficient to overcome the positive G1 exit de-block function of the phosphorylated Rb protein in G1/S boundary (see paragraph "Tumor suppressor genes") (11).

Interferones α, ß and γ all inhibit moltiplication both of normal and transformed cells. They seem to inhibit cell proliferation without concurrent inhibition of macromolecular biosynthesis.

Particularly, IFNß seems to decrease the expression of some of those genes that are activated at the transcriptional or post-transcriptional level when growth is induced with growth promoting factors (12).

IL-1 is an inhibitor of endothelial growth in vitro and in vivo. It arrests ongoing cells in G1. In an angiogenesis model, IL-1 could inhibit FGF induced vessal formation. A mechanism by which IL-1 may exert its inhibitory effect on growth was suggested by the fact that IL-1 decreased the expression of high affinity FGF binding sites on endothelium (13).

Another peptide that possess growth inhibitor activity is the Mullerian inhibiting substance. It is found exclusively in mammalian testis. It causes regression of the Mullerian ducts during the development of the reproductive tract in male embryos.

In addition, some glycosamminoglycans, such as heparin, have been shown to be potent inhibitors of the proliferation of several cell types, including vascular smooth muscle cells, renal mesangial cells, certains types of fibroblasts and cervical epithelial cells.

Heparin is able to decrease the number of EGF receptors on calf smooth muscle cells and alters the profile of matrix associated and secreted proteins. It suppresses the induction of c-fos and c-myc mRNAs by serum while it has no effect on mitogenic response to EGF in NIH3T3. Infact it seems that heparin inhibits proto-oncogene expression and cell proliferation by blocking one or more steps in PKC dependent pathway (14).

5. Growth arrest specific gene expression:

If quiescence is a real different metabolic state of the physiology of the cell, it must be the phenotipic effect of a peculiar pattern of gene expression.

From this point of view, the isolation of genes specifically expressed in this phase can be very interesting and help us in the comprehension of the differences between a resting and growing cell.

The first identified marker of quiescence was Statin. It is a 57 Kd protein present only in nonreplicating cells and down regulated after 6 hours from serum addition (15).

Another mRNA specifically expressed in quiescence was cloned in chicken embryo fibroblast (CEF). It appears to be tightly down regulated by EGF and Insulin (6 hours after addition), by activation of a ts mutant of $pp^{60v\text{-}src}$ and induced by density-dependent inhibition. It codes for a growth arrest specific protein that is secreted and it has a certain homology to plasma retinal binding protein (16).

In NIH3T3 model system, the screening of a substractive cDNA Go specific library has resulted in the

isolation of 6 genes that have a high level of expression in quiescence and a low one in activated cells (17).

These six genes were named gas genes (growth arrest specific).

All the six genes were also induced by density dependent inhibition.

The decrease of the gas mRNA starts after 1 hour and is maximal by 6 to 10 hours after addition of serum corresponding to the mid and late G1 phases.

With the exception of gas2 and 3 mRNAs, gas mRNAs reappear again between 12 and 18 hours after serum addition.

Addition of Cycloheximide during the activation do not prevent the decrease in gas mRNAs suggesting the lack of involvement of the immediate early growth factor response in this process (18).

Gas1 is the only gas gene regulated at transcriptional level.

The mechanism of the post-transcriptional control involved in the deregulation of the gas mRNA is still unclear. The half lives of the mRNA were around 2-3 hours for gas1 2 3 6 and around 6 hours for gas5. Moreover the turnover of these mRNAs in serum-stimulated cells is slower than that observed in resting cells.

Expression of gas mRNA has been detected in several fibroblast cell lines and also in primary embryo fibroblasts. They are, however, also expressed in other cell types, in different tissues and during embryo development.

Gas1 is not only down regulated by serum but, in the uterus of ovariectomized rats seems to be under negative control of estrogen hormones.

Gas gene products have shown that a resting cell has some peculiar features in all its compartments: from immunofluorescence data, it seems that gas2 protein is associated to the cytoskeleton; sequence analysis and in vitro translation experiment in presence of microsomes suggest that gas3 codes for a membrane protein (19) and gas6 codes for the murine analog of protein S, a secreted protein involved in the regulation of coagulation.

6. Tumor suppressor genes:

When somatic cell hybrids are produced by fusing a tumorigenic cell with a non-tumorigenic cell in culture, the resulting hybrid cell line is most often non-tumorigenic.

This appears to be correct independently of the etiology of the tumorigenic cell line and independenly of the cell lineage of the tumorigenic cell line (epithelial cell, fibroblast or lymphocyte).

These hybrids cell lines, when inoculated into animals to test for tumor production, sometimes do produce tumors. In most of the cases, tumor formation has become associated with the loss of a specific chromosome derived from the non-tumorigenic parent.

These data have led to the suggestion that the non-tumorigenic parent supplies tumor suppression gene function that have been localized by these experiments to several human or mouse chromosomes.

Furthermore, different tumors may have lost or inactivated different tumor suppression genes (20).

The normal allele of an oncogene is involved in the positive network of regulation of cell growth (growth factor receptors, protein kinases, nuclear protein), tumor suppressor genes can be involved in the normal negative control: the lack of the capacity of the cell to interpret negative growth control signals can have comparable dramatic effect on cell proliferation (21).

In human tumors, for example, deletions in chromosomes 11p and 13q are found in breast cancers, while 3p, 13q and 17p are found to suffer deletions in lung cancers and 5q, 17p, 18q and 22q are all found to be altered in many colorectal carcinomas.

The childhood malignacy retinoblastoma has proven to be an essential model in the development of the theory of recessive genes. 20 years ago, Knudson proposed that both the inherited and spontaneous forms of retinoblastoma resulted from only two separate genetic events that would result in the mutation or inactivation of two genetic loci. Familiar cases would result from the acquisition of a germ line mutation as a primary event and a somatic mutation of a second allele would be the mutation ultimately responsible for the expression of tumorigenicity. In spontaneous retinoblastoma both events would occur somatically.

The Rb cDNA was cloned two years ago (22). It has no homology with any particular sequence motif. When it was cloned in a retroviral expression vector, it was able to suppress the tumorigenicity of a human retinoblastoma cell line, osteosarcoma cell line (23) and three human prostate carcinoma cell lines that had deleted 105 nucleotides encoded by exon 21 (24).

The mouse Rb homologue encodes a protein that is 90% identical in its aminoacids sequence to its human counterpart.

The Rb gene product is a phosphoprotein localized in the nucleus and its phosphorylation state is modulated during normal cellular events.

In resting cells, Rb protein is present in its less phosphorylated form; in rapidly proliferating cells, Rb protein is highly phophorilated. The phosphorylation event occurs at the late G1/S boundary (25).

Induction of differentiation in several human leukemia cell lines by treatment with phorbol ester or retinoic acid leads to dephosphorylation of Rb. Time courses studies indicate that Rb dephosphorylation precedes the total arrest of cell growth during differentiation (26).

For in vitro assays, Rb results as a DNA binding protein. Recently it was shown that is able to bind c-fos promoter and to down-regulate the expression of a reporter gene at the 3' end.

The Rb protein forms complexes with SV40 LT, the transforming protein of SV40 virus, adenovirus E1A protein,

which is required for adenovirus-induced cell transformation and E7 protein of human papillomavirus (HPV), which is required for HPV-induced tumor formation (27) (28) (29).

While E1a is able to bind to both of the different phosphorylation states of the Rb protein, SV40 LT is able to bind just to the Go-G1 specific form. The region of E1a and SV40 LT involved in immortalization and transformation are the same that in Rb binding suggesting that this interaction can be a crucial point in the mechanism of transformation adopted by these viruses. Meantime the Rb domain involved in the binding to the viral proteins is exon 21 that is found to be highly mutated in tumorigenic cell lines. In the transgenic mice that, expressing the SV40 LT antigen, developed a retinoblastoma phenotype in the retina, a close interaction between this protein and the Rb gene product was found conferming in vivo the in vitro results (30).

From these data it's clear that the antioncogene-related transformed phenotype of a cell can achieved by different mechanisms:

-deletion of both the alleles
-mutation in the promoter
-mutation in the active sites of the protein
-inactivation of the protein

Bearing in mind that tumor suppressor genes probably have a key role in the inhibition of cell proliferation in normal cell growth, the underphosphorylated form of Rb protein can be able to confer a block on the entry in S and the inactivation of this block via phosphorylation is one of the key regulatory points in the entry in S phase. This mechanism could be also controlled via negative growth factors as TGF-ß1.

The short arm of the chromosome 17 is often involved in the development of many human tumors.

The p53 gene maps in this locus.

In the majority of human colon carcinoma with allelic deletions of chromosome 17p, the remaining p53 allele contains a missense mutation. This gene has also been found mutated in tumors of the brain, breast, lung and bone (31) (32) (33).

These studies are consistent with the hypothesis that the normal p53 gene product may function as a suppressor of neoplastic growth and that inhibition or deletion, or both, of the wt gene inactivates this suppression (34) (35) (36).

The gene is composed of eleven exons. The gene encodes a 2.2-2.5 Kb mRNA producing a protein of 53 Kd. The primary aminoacid sequence of this protein can be divided into three distinct domains: an acidic NH_2- terminal, that forms an alpha helical structure, an extended proline rich, hydrophobic region and a COOH- teminal basic domain with helix coil helix motifs and the ability to bind DNA.

From a biochemical point of view, it has been recently found that the protein encoded by the wt p53 fused to GAL4 DNA binding domain is able to activate transcription of a reporter gene while the mutated form is not.

Meantime it has been shown that wt p53 can inhibit the replication of SV40 DNA in vitro. The mutated form is not able to do it.

The mouse and human proteins share 80% homology at the aminoacid level. The aminoacid sequence of the human, mouse and Xenopus p53 proteins show five regions of extensive aminoacid conservation (90-100%).

Actively growing cells contain 5 fold higher levels of p53 mRNA. In most transformed or tumor cells, p53 protein levels are elevated from 5 to 100 fold above its normal cell counterpart. In the majority of these cases, this is due to an increased of half-life of the p53 protein itself.

It has been shown that the expression of p53 wt is able to suppress the ability of E1a plus ras or c-myc plus ras to transform primary rat embryo fibroblast. Moreover, wt p53 is able to inhibit the growth of colorectal carcinoma cell lines, gliobastoma tumor cell lines and SV40-transformed hamster cells (37) (38) (39).

There is little p53 in cells that have just completed mitosis and newly synthesised protein is expected to accumulate in a underphosphorylated state during early G1.

The greatest incorporation of phosphates into p53 occurs in G1/S boundary and it seems to be a substrate for p60-cdc2 and cyclin B-cdc2 kinase activities (40).

In SV40 transformed cell lines, in adenovirus transformed cell lines and in papillomavirus transformed cell lines, the p53 protein was found complexed with viral oncogenes products: SV40 LT, E1B and E6 respectively. These complexes result in an increased p53 protein half-life.

Cytogenetic analysis has identified chromosome 11p13 as the smallest overlap region for deletions found in individuals with WAGR syndrome which includes Wilms tumor, aniridia, genito-urinary abnormalities and mental retardation .

Recently, by chromosome walking techniques, it was shown that the gene responsible for this pathology codes for a protein that contains four zinc-finger domains and a region rich in proline and glutamine, features that suggest a role in transcriptional regulation (41) (42) (43).

Allelic deletions involving chromosome 18q occur in more than 70% of colorectal cancers.

Recently the gene involved in this pathway was cloned.

The predicted aminoacid sequence of the cDNA codes for a protein with sequence similarity to neural cell adhesion molecules and other related cell surface glycoproteins.

Probably this protein is involved in the cell-cell interactions that control growth.

A more direct approach in order to clone new tumor suppressor genes was made transfecting cDNA lybraries of normal cells in transformed cells and analyzing the revertant clones.

This strategy was very useful in cloning the Krev-1 gene that is able to suppress the K-ras transformed phenotype. It has been shown that this gene is highly related to K-ras, maintaining its biochemical properties (44)(45).

A very exciting link between these two approaches had been with the cloning of the gene responsible for the

Neurofibromatosis Type 1 (NF1), a genetic desease with autosomal dominant inheritance.

The cloned cDNA fragment shows a striking homology to GAP proteins and IRA1 gene (46).

GAP is able to bind ras protein and it is able to induce at first the effector cascade of ras action and then the hydrolisis of GTP that inhibits the transmission of the signal.

Krev-1 protein is able to interact with GAP in the same domain of ras protein without any activation of the positive signal: by this mechanism it is a competitor of ras.

IRA1 is an inhibitor of yeast ras1 function and it is able to stimulate its GTPase activity.

The NF1 gene product may interact directly or indirectly with the product of a ras or ras-like protein to inhibit growth-stimulating activity in target cells.

Failing to provide this inhibition, the mutated NF1 gene product could favourite an inappropriate growth in target cells.

7. Exit from quiescence:

Cycling cells require about 6 hours after mitosis to enter S phase, while quiescent cells require at least 12 hours.

Infact if cycling cells are starved for two hours and then again with high serum the entry in S phase is delayed of 8 hours: it means that the re-entry in cycle from quiescence needs 6 hours and that there are some peculiar biochemical and genetic events that trigger this particular transition from the "out of cycle" state to the "in cycle" one.

During this transition, resting cells must overcome some control points that define different subphases depending on the effects of limiting growth factors and nutrients inhibitors as measured by the time needed to reach S phase after the block is removed.

These subphases are named competence, entry, progression and assembly and are separated by C, V and R points.

The first step is the "competence". Untrasformed BALB/C 3T3 mouse fibroblasts do not progress to S from Go if they are provided with either PDGF or plasma. If they are first given PDGF briefly and subsequently provided with plasma, they progress to S but not viceversa. Thus, these PDGF-treated cells carry out some initial processes called competence. They require as much time to reach S phase as do Go cells.

Competent cells progress to S the phase after they are given plasma, which contains other factors such as EGF and Insulin. Competent cells incubated with plasma in a medium lacking essential aminoacids move towards S up to a point named V. After the aminoacids are provided, the cells at the V point require only 6 hours to reach S phase. This

lenght of time is very similar to the duration of G1 for the cycling cells.

The only growth factor required (by 3T3 cells) to progress to S after the V point is IGF-1. This further progression requires net protein synthesis (47).

8. Positive growth factors:

From a molecular point of view the response of the resting cells to the activating signals is at different levels.

Some effects are "immediate" being detectable within seconds or minutes of growth factor addition. These changes include rapid ionic changes, cytoskeleton redistribution, protein phosphorylation, membrane lipid metabolism changes and alteration in the protein synthetic machinery. Other effects are not so rapid and are called "early events", they include induction of specific new gene transcription and expression of new protein.

The first determining event in the metabolic cascade is the interaction between the growth factor and its receptor. Ligand binding and the consequent conformational allosteric changes of the extracellular domains induces receptor oligomerization, which stabilizes interactions between adjacent cytoplasmic domains and leads to the activation of kinase function by molecular interaction (48).

It is assumed that the phosphorylation of cellular substrates, together with alterations in the ionic content of the cell, provides an internal stimulus for cell growth (49).

However the chain of events that is initiated by the phosphorylation of cellular substrates is still poorly understood. One possible pathway involves the phosphorylation of phospholipase C-γ. The activation of phospholipase C-γ leads to the generation of phoshoinositol metabolit such as IP_3, which causes the release of Ca^{++} from intracellular compartiments, and the generation of diacylglycerol (DAG) that activates the Protein Kinase C (PKC). Microinjection of exogenous PLC-γ into NIH3T3 cells overwides the cellular Go block and induces DNA synthesis showing that PLC-γ is a key enzyme in one of the activation pathways of the entry in S phase.

A second possible pathway for the activation of the entry in S phase is mediated by cAMP. Some polipeptide hormones can exert their effect by either activating or inactivating the inner plasma membrane-bound enzyme adenylate cyclase. This alters the level of cAMP inside the cell which then determines the activity of another set of kinases as the cAMP-dependent Protein Kinases. Apparently, different cells can express different sets of kinases or different sets of target proteins, as a consequence changes in cAMP levels can have different effects in different cell types. The effect of cAMP is assumed to be mediated through

PKA activation and phosphorylation of specific protein substrates. It involves the activation of c-myc and c-fos, even if the induction of c-myc expression is not sufficient to promote all the cAMP-mediated induction of DNA synthesis. The other events are: the induction of ornithine decarboxilase activity, poliammine synthesis by TSH and an increase in ras proto-oncogene mRNA content.

Different growth factors are able to induce different metabolic pathways that can be entirely distinct at the level of membrane signalling and protein kinase activation, partially overlapping at the level of gene expression and protein synthesis and are believed to converge on DNA synthesis.

Comparison of biochemical events associated with the different pathways leading to proliferation should allow the dissection of obligatory events that are common to all the pathways and help to discriminate between events that regulate the cell cycle and those that are regulated by the cell cycle.

Moreover the detection of the distinct mitogenic pathways can be coupled with different pattern of differentiation.

In vivo PDGF is a major factor for connective tissue cells. Structurally it is a dimer with two different polypeptide chains. All the three dimeric combinations have been identified and purified from platelets and transformed cells.

PDGF is able to activate the PLC-γ dependent pathway and in this way activates the PCK.

Overexpression of PLC-γ in transfected cells leads to a dramatic overproduction of IP_3. The PDGF induced Ca^{++} signal and DNA synthesis were not increased in the PLC-γ overexpressing cells. Moreover using an inhibitor of Tyrosine Protein Kinase it has been shown that PDGF is able to induce DNA synthesis without the occurence of PLC-γ activation. These data suggest that PDGF-induced IP_3 generation is not the unique mechanism underlying PDGF-induced DNA synthesis (49) (50).

EGF is mitogenic for a variety of mesenchimal cells and epithelial cells in culture.

It consists of a 6 Kd single chain with three intrachain disulphide bonds. The nucleotide sequence of a complete cDNA predicts a large protein precursor of 1217 aa with mature EGF in the -COOH end.

It binds to its own receptors inducing dimerization and phosphorylation of its own polypeptide chain and of exogenous substrate. Mitogenic stimulation of quiescent mouse 3T3 cells with EGF triggers a lot of cellular responses in common with PDGF. It is able to induce Tyrosine phosphorylation of PLC-γ, Ca^{++} uptake into cells, the activation of glycolisis and ornythine carboxylase.

Moreover EGF is able to activate a pathway that involves the phosphorylation on Serine and Threonine residues. This effect is mediated by two proteins: the S6 protein kinase and the S6 kinase kinase. The S6 kinase

utilizes ATP to phosphorilate the 40S small ribosomal subunit S6, and this event is necessary for the initiation of new protein synthesis which presumably increases the supply of proteins essential for growth.

Detailed kinetics have revealed that EGF-induced S6 kinase activation is biphasic: an early phase appears after 15' followed by a late phase between 30' and 60'.

Direct activation of PKC by TPA induces just the late phase while down regulation of PKC doesn't affect the early phase of EGF increased response. The loss of late phase results in decreased EGF induced S6 phosphorylation, protein synthesis and cell growth. So EGF differentially regulates S6 kinase activation by two direct signalling pathways one PKC dependent and the other one not.

The signalling pathways regulating the first phase of S6 kinase has not been yet identified. There are two possible candidates: MAP-2 Kinase and c-raf proto-oncogene.

In NIH3T3 EGF utilizes the cAMP pathway to activate c-myc and c-fos. This pathway acts through cyclic AMP-dependent protein kinase (PKA).

In other system, canine thyroid cells, EGF activation is completely distinct from the cAMP and PKC pathways.

EGF is also able to activate the Casein Kinase II that is able to phosphorilate acidic protein substrates. Casein Kinase II utilizes extra ATP or GTP and is highly sensitive to inhibition by heparin or stimulation by poliammines. As substrates it has c-myc, p53, enzymes as RNA pol II, protein phosphatase and nucleolin. It is able to phosphorylate the nuclear c-myb protein in vivo and in vitro and this event is able to inhibit the sequence-specific binding of c-myb to DNA. Infact this site is deleted in nearly all oncogenically activated myb proteins resulting in DNA-binding that is indipendent of CKII activity (51) (52).

There are potential sites also in E1A and SV40LT.

bFGF is a potent mitogen for a wide variety of cell types of mesodermal and neuroectodermal origin. When supplied exogenously to tissues of living organisms such as the chorioallontoides membrane of the chick embryo, bFGF is able to induce angiogenesis (53).

More recently a role for bFGF in the induction of mesodermal tissues has been demonstrated during the normal development of the embryo (54).

In Go-arrested Chinese hamster lung fibroblasts, the bFGF receptor signaling pathway is not coupled to PLC-γ activation. In this system, the early mitogenic events such as DNA synthesis are activated independently of the generally accepted pathways for the activation of growth factors.

In 3T3-L1, bFGF may exert some of its effects through at least two distinct pathways, one dependent and the other independent of PKC. In G1 arrested aortic endothelial cells, addition of bFGF induced the transition G1-S and the growth factors is then detected in the nucleus.

Recently it was shown that even if cellular uptake of bFGF occurs continously during the cell cycle, its entry into the nucleus is stage specific.

Insulin and the insulin like growth factors IGF-I and IGF-II are three homologous peptides that bind with different affinities to three different receptor types. Insulin has two disulfide bonded chains; it is able to stimulate glucose uptake, protein synthesis and lipid synthesis. It can mimic the action of IGF-I.

9. Go-G1 transition specific gene expression:

When the activating signals, mediated by second messengers or directed, reach into the nucleus, a dramatic change in gene expression takes place. In particular, three different stages can be detected (55) (56).

An early immediate gene activation leads to the expression of a series of genes after 30' from the addition of serum. These genes are tightly down regulated to undetectable levels just 1 hour and half later. Two examples of these genes are c-fos and c-jun.

Another set of genes appears after 2 hours of stimulation and slowly decrease therafter. C-myc belongs to this set of genes. The half lives of the mRNA of these genes is very short (15'-30').

The last group codes for components of cytoskeleton or extracellular matrix. They appear after 2 hours of stimulation and remain expressed for 8 hours more. Their mRNAs have a very long half lives. Examples of this set of genes are ß-actin, ß subunit of fibronectin receptor, fibronectin and tropomyosin.

Totally 80 genes seem to be activated during Go-G1 transition and they can resemble all the possible motifs present in different proteins: Krox 20 is a transcriptional activator that contains a zinc-finger motif, clone 482 is a membrane protein that is the murine analog of human Tissue factor; vimentin is an intermediate filament protein that is part of the cytoskeleton; calcyciclin is a Ca^{++} binding proteins and clone 2F1 is an ATP/ADP tranlocase. Plasminogen activator inhibitor and another human Tissue factor analog are secreated proteins. Most of the genes are regulated at transcriptional level and their induction is cycloheximide insensitive.

The transcription of some genes is activated later in Go-S transition. This process is sensitive to cycloheximide and requires that essential aminoacids be brought into the cell by specific transport systems.

Few studies have identified mRNAs and proteins that appears in mid or late G1. The ras gene can be activated in mid-G1; its role here is indicated because antibodies to ras protein blocks progression if it is incorporeted in the first but not in the latter half of G1.

Another gene that seems to be involved in the control of late G1/S transition is c-myb. Infact the inhibition of its expression by antisense oligosis able to block the entry into S phase in normal human T-lymphocytes but not c-myc induction and all the other immediate early events.

MATERIALS and METHODS

1. Cell lines and cell culture conditions:

NIH 3T3 cells were kindly provided by Dr. R. Muller.
They were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum FCS, penicillin and streptomycin (100 U/μg/ml).
For serun starvation, the medium was changed to 0.5% FCS when the cultured cells were subconfluent. The cells were then left in the same medium for 48 hours. Under these conditions, incorporation with 50μM BUdR for an additional 24 hours resulted in labeling of less than 3% of the nuclei.
For the induction of DNA synthesis, fresh medium containing 10% to 20% FCS was added to the arrested cells.
The cells were then harvested at the desired times for RNA isolation. After 24 hours of BUdR incorporation, more than 90% of the nuclei scored positive.
In some experiments, the entry in DNA synthesis has been induced adding a single growth factor at the concentration of 20ng/ml of PDGF, 100ng/ml of FGF, 20ng/ml of EGF and 100μg/ml of insulin.
For density-dependent inhibition, cell were plated at $10^4/cm^2$ in 10% FCS. The medium was changed twelve hours after plating, and this operation was repeated every 2 days. After 4 days in culture, incubation with BUdR for 2 hours resulted in less than 1% incorporation. When used, Cycloheximide and Actinomycin D concentrations were, respectively, 10 and 5μg/ml.

2. DNA synthesis assay:

Cells grown on cover slips (20 by 20 mm) in the same culture dishes from which RNA was prepared were incubated for 2 hours in the presence of 50μM BUdR. After this time they were fixed for 5' in methanol at 4° C and then for 5' in acetone at 4° C. DNA was then denatured by treatment with 1.5N HCl for 10'. The cover slips, after washing with phosphate-buffered saline (PBS), were incubated with mouse monoclonal antibody against BUdR for 1 hour at 37° C, washed three times in PBS and then incubated for 45' at 37° C with tetramethylrhodamine isothiocyanate (TRITC) conjugated rabbit antimouse immunoglobulin antibodies. Total nuclei were visualized with Hoechst stain 33342 (1μg/ml). More than 300 nuclei were observed for each cover

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

3. Total RNA preparation:

Total cellular RNA from cell cultures was prepared by washing the plates twice with PBS, followed by addition of lysis buffer (guanidine thiocyanate 4M, Na-citrate 25mM, ß-mercaptoethanol 100mM, N-lauroylsarcosine 0.5%, antifoam 0,1%). After the homogenization in Polytron, Sodium acetate pH 4.0 was added to a final concentration of 0.2M. Then one volume of phenol/water and 0.2 volumes of chloroform were added. After an incubation in ice, the mixture was spin at 10000 r.p.m. for 15' and the supernatant was mix with one volume of isopropanol and left O/N at -20° C. After spinning, the pellet was solved in 0.25 of initial volume of the lysis buffer and spin again. To the supernatant two volumes of ethanol were added and the tube were left O/N at -20° C. After precipitation and washes in ethanol 70%, the RNA was resuspended in water.

The murine organs were conserved at -80° C.

4. Poly(A)$^+$ RNA preparation:

The poly(A)$^+$ fraction of total RNA was prepared using the Stratagene's columns that contain an oligo(dT) cellulose. The total RNA was pre-heated at 65° C for 5' and put quickly in ice. Then the RNA was diluited in the sample buffer (Tris-HCl 1mM pH 7.5, EDTA 1mM pH 8, NaCl 0.5M final). After the equilibration of the column in high salt buffer (Tris-HCl 10mM pH 7.5, EDTA 1mM pH 8, NaCl 0.5M) with two washes, the RNA was applied to the column and push thorough at a rate of approximately one drop every two seconds. Then the eluted solution was re-applied. The column was washed twice with high salt buffer and three times with low salt buffer (Tris-HCl 10mM pH 7.5, EDTA 1mM pH 8, NaCl 0.1M). At the end the mRNA was eluted with the pre-heated eluition buffer (Tris-HCl 10mM pH 7.5, EDTA 1mM pH 8).

5. Construction of a cDNA library:

The cDNA library was constructed using 3 µg of poly(A)$^+$ mRNA extracted from quiescence cells.

The first strand cDNA synthesis was performed preparing the following mixture: oligo(dT) 200ng/µl 5 µl, reverse transcriptase buffer 5x (Tris-HCl 250mM pH 8.3, KCl 375mM, dithiotreitol 50mM, $MgCl_2$ 15mM) 8 µl, RNAsin (Promega) 35 U, hot [^{32}P]dCTP 1µl, dNTP 10mM 4µl, H_2O 13µl. The RNA was pre-heated 3' at 68° C and cool in ice. Then it

was added to the reaction mixture together with 1 μl of M-MLV Reverse Transcriptase 200U/μl (BRL). The reaction was performed for 1 hour at 37° C.

The second strand synthesis was performed in this conditions: H_2O 124 μl, II strand buffer 4x (Tris-HCl 80mM pH7.5, $MgCl_2$ 16mM, ß-mercaptoethanol 60mM, KCl 400 mM) 60 μl, BSA (Promega) 2.5μg/μl 4.5 μl, NAD^+ 15mM 2.4 μl, DNA polimerase I (Pharmacia) 6 μl, RNAse H (Pharmacia) 1 μl, Ligase E.coli (Pharmacia) 15 μl for 2 hours at 16° C.

Then the reaction mixture has been loaded on a G100 Sephadex equilibrated in water. The labeled aliquots were pooled and dried to 68 μl. To this solution 10 μl of T4 polimerase buffer 10x (Tris-HCl 670mM pH 8.8, MgCl2 67mM, ß-mercaptoethanol 100mM, ammonium solphate 166mM, EDTA 67μM pH 8, BSA (Promega) 1.6mg/ml) was added together with 15 μl of water, dNTPs 10mM 1 μl and 2 μl of T4 DNA polimerase (Pharmacia). The reaction was performed for 30' at 37° C.

Then a phenol/chloroform and a chloroform extraction was performed.

The solution was loaded on an A5 cellulose column equilibrated in Tris-HCl 1mM pH 7.5, EDTA 0.01mM pH 8. The labeled eluted fractions were pooled and dried to 19 μl. This aliquot was added to a solution containing 3 μl of EcoRI adaptors, 6 μl of ligase buffer 5x (Tris-HCl 250mM pH 8, $MgCl_2$ 35mM, DTT 5mM, rATP 2.5mM), 2 μl of T4 DNA Ligase (Pharmacia) and incubated O/N at 4° C. The solution was diluted to 20mM final EDTA pH 8 and loaded on 1% agarose gel. Just the cDNA fraction longer than 2 Kb was recovered by the gel using Glass-beads from Bio-rad. To the recovered 18 μl solution was added 1 μl of rATP 100mM, 5 μl of Ligase buffer 5x and 1 μl of T4 polynucleotide kinase (Pharmacia). The mixture was incubated at 37° C for 30'. Then a phenol/chloroform and a chloroform extraction was performed and the last upper phase was loaded on an A5 cellulose column equilibrated in Tris-HCl 1mM ph 7.5, EDTA 0.01mM pH 8. The labeled fractions were pooled, dried to 6 μl and ligated with 2 μl of EcoRI cut and dephosphorylated lambda G vector 0.5 μg/μl. After adding 2 μl of T4 Ligase buffer 5x and 1 μl of T4 Ligase (Pharmacia), the mixture was incubated O/N at 4° C. The day after half ligation mixture was packaged in Stratagene packagene extracts and plated infecting E.coli NM554 cells.

6. Hot cDNA screening:

At first the phage's DNAs were transferred onto a Genescreen nylon membrane. The filter was put on the agar plate for 1' and then immediately transfer on a solution containing NaOH 0.5N for 2'. The denaturation step is repeated once more and then the filter is neutralized with Tris-HCl 0.5M pH 7.5. The neutralization step is repeated once more. Then the DNA is cross-linked by exposure to UV light (Statalinker,Stratagene). Pre-hybridization was performed in 15 ml of NaCl 1M, SDS 2%, Magic Juice 100μg/ml

(bacterial and phages DNA), salmon sperm DNA 100μg/ml. The hybridization was performed in the same buffer with the addition of 2.5mg/ml of heparin and of hot cDNA. The probes were prepared with the synthesis of hot first strand cDNA. 250 μCi of [^{32}P]dCTP were liophilized to 1 μl. Then it was added 1 μl of water, 0.8 μl of Reverse Transcriptase buffer 10x (Tris-HCl 500mM pH 8.3, KCl 750mM, dithiotreitol 100mM, $MgCl_2$ 30mM), 0.8 μl oligo(dT), 0.8 μl dNTPs 10mM, 1μl dCTP 75μM. 100 ng of mRNA poly(A)$^+$ was pre-heated 3' at 68° C and cool immediately in ice. Then it was added to the reaction mixture together with 1 μl of M-MLV Reverse Transcriptase 200 U/μl (BRL).The reaction was incubated 1 hour at 42° C. Then the solution is diluted to 90 μl with EDTA 20mM final. 10 μl of NaOH 2M was added and put at 68° for 20'. Then 10 μl of Tris-HCl 1M pH 8 and 19 μl of HCl 1M were added. Equal amount of c.p.m. were added to the hybridization solution for different cDNAs synthesis (~500000 c.p.m./ml). The hybridization was performed for 30 hours at 65° C. The washes were: SSC 2x room temperature twice for 15'; SSC 2x, SDS 1% twice 65° C 30'; SSC 0.2x, SDS 0.1% twice 50° C 10'; SSC 0.1x, SDS 0.1% twice 50° C 10'. Then the filters were left to dry and exposed three days at -80° C.

7. DNA sequencing:

DNA fragments were sequenced directly in lambda G vector using the T7 sequencing kit. The products of the sequence reactions were separated in a 6% polyacrylamide gel which contains urea 8M final and TBE buffer 10x(Tri-borate 890mM, boric acid 890mM, EDTA 20mM pH 8).

8. In vitro transcription and translation:

The lambda G vector was linearized downstream from the T7 promoter with HindIII. 1 μg of linearized lambda was incubated for 60' at 37° C in Tris-HCl 40mM pH 8, $MgCl_2$ 8mM, NaCl 50mM, ATP, CTP 1mM, UTP 0.2mM, GTP-cap analog 1mM, dithiothreitol 10mM, RNAsin (Promega) 35 U, T7 RNA polimerase (Stratagene) 20 U in a total volume of 20 μl.The DNA template was removed by adding 15 U of RNAse free DNAse (Pharmacia), followed by incubation at 37° C for 15' in a 40 μl of volume. RNA was then purified as described for the nuclear run-on assay. After extraction by phenol-chloroform, the RNA was precipitated with 2 volumes of ethanol. Translation reactions were performed by using rabbit reticulocyte lysate (Amersham). Approximately 1 μg of RNA was translated in a 25 μl reaction volume containing 50 μCi of [^{35}S]methionine at 30° C for 60'. The translation products were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE). For fluorography, the gels were fixed in 45% methanol-7% acetic acid for 1 hour and then

treated with En3Hance (Dupont). The gels were then rinsed with water, dried and exposed for autoradiography at -80° C for 1 to 3 days

9. Northern blot analysis:

Total cellular RNA (10 μg) was used for Northern analysis on 1% agarose gel containing 6.7% formaldehyde. Integrity and relative amount of RNA were analyzed by ethidium bromide staining. Gels were transferred for 16 hours to a Duralon-UV nylon membrane (Stratagene). RNA was cross-linked by exposure to UV light (Stratalinker, Stratagene). Hybridization was performed in NaCl 1M, SDS 1% at 65° C using $5x10^5$ c.p.m. of probe per ml, prepared by random primer synthesis (Boehringer). Washes were performed as follows: SSC 2x room temperature twice 5'; SSC 2x, SDS 0,1% 65° C twice 15'.

10. Run-on experiment:

For the isolation of nuclei, cells were washed twice with iced PBS and taken out. After a gentle spinning, the pellet is incubated for 5' in lysis buffer (Tris-HCl 10mM pH 7.4, NaCl 10mM, $MgCl_2$ 3mM, NP40 0.5%). Then the lysate is centrifugated again, the pellet is resuspending in the storing buffer (Tris-HCl 10mM pH 8.3, glycerol 40%, $MgCl_2$ 5mM, EDTA 0.1mM pH 8) and stored in liquid N_2.

For the run-on transcription assay, 100 μl of the nuclear suspension was mixed with 100 μl of reaction buffer (Tris-HCl 10mM pH 8, $MgCl_2$ 5mM, KCl 300mM, ATP, CTP and GTP 0.5mM each, [^{32}P]UTP 150 μCi) and incubated for 30' at 30° C. The [^{32}P]-labeled RNA was then isolated by passage through a Sephadex G-50 (Pharmacia) spun column equilibrated in TLES (Tris-HCl 10mM pH 7.5, LiCl 0.1M, EDTA 0.1mM pH 8, SDS 0.05%). The DNA was spotted onto a nylon membrane (Stratagene); 10 μg of previously denatured DNA (NaOH 0.25M for 20' at room temperature, neutralized by addition of an equal volume of SSC 0.25x) was applied per slot. Hybridization was performed in NaCl 1M, SDS 1%, heparin 1mg/ml, salmon sperm DNA 100μg/ml at 65° C per 36 hours with a probe concentration of $2x10^6$ c.p.m./ml. The filters were then washed with SSC 2x at room temperature twice for 10'; with SSC 2x, SDS 1% at 65° C for 15', and finally with SSC 0.2x, SDS 0.1% at room temperature for 10'.

RESULTS

1. Isolation of the clone:

Total RNA has been extracted from NIH3T3 cells that were starved in 0,5% FCS for 48 hours.

Using a poli(dT) column, I have isolated the poly(A)$^+$ fraction of the mRNA.

Starting with 3 μg of this poly(A)$^+$ mRNA, I have constructed a cDNA library cloned into the EcoRI site of lambda G.

The library consisted of 100000 clones.

Being my interest the characterization of genes specifically expressed in quiescence, I decided to screen this library with hot cDNA synthetized from mRNA poly(A)$^+$ of resting cells and of cells after 6 hours of addition of 20% of serum.

I plated the library in 5 plates and I transferred the phage's DNA into a nylon Gene Screen membrane twice.

Using 100 ng of poly(A)$^+$ mRNA and 250 μCi of [^{32}p] labeled dCTP, I got 10^7 c.p.m. of labelled first strand cDNA.

Then I used this two hot cDNAs as probes for the hybridization with filters that contain the cDNA library.

The hybridization and the washes were performed as describes in "Materials and Methods" section.

After three days of exposure, I was able to pick up 112 not purified clones that seemed to be more expressed in quiescence.

Then I plated a serial dilution of each single region of plaques that corrisponded to the spot on the autoradiography.

I transferred the diluted plaques into the nylon membrane and hybridized them again with the two hot cDNAs.

After this screening I have got 96 purified clones that seemed to be more expressed in quiescence.

In order to see if some of these clones correspond to the already cloned gas genes I grew separately all the 96 clones in a single plate and transferred them into a nylon membrane.

Then I hybridized it with probes containing parts of the cDNA of some gas genes.

15 plaques seemed not to cross-hybridized with cloned gas genes.

At the restriction and Southern analysis they appeared to be cDNA of different lenghts of the same mRNA.

The longest clone was 3,1 Kb. It was named gas7 (growth arrest specific) and further characterized.

2. First characterization of the sequence:

Using the primer T7 and T3 that are present in the vector, I made some sequence reactions in lambda in order to see if this cDNA has been already cloned or resembles the sequence of a family of genes. I sequenced 300 bp at the 3' end and the same at the 5' end and I made a computer research against EMBL and NIH DNA sequences bank data. At the 3' end I found the poly(A) tail.

No significative homology was found. I concluded that gas 7 is a new unknown gene.

Then I subcloned some restriction fragments into a plasmid (bluscript) in order to sequence the whole clone.

3. Transcription/Translation analysis:

In order to see if the gas7 clone is able to code for a protein in vitro, I decided to transcribe it and then translate it.

At first I cut the lambda clone of gas7 with HindIII that recognizes a site of the polilinker of the lambda DNA at the 3' end of the cDNA.

Then I transcribed the cDNA into a capped RNA, 3,1 kb long, using T7 RNA polimerase and the T7 promoter.

After having purified the RNA, I translated the in vitro synthetized RNA in a reticulocyte lysate and run a 8% polyacrilamide gel in order to see if a protein has been translated and how long it is.

In photograph 1 it is possible to see the in vitro translational product of gas7: it is ~75 Kd long.

From this analysis it comes out that the clone of gas7 I got from my screening contains an ORF long ~2 Kb and has an active translational initiation site (figure 1).

4. Pattern of expression of gas7 mRNA after serum addition:

The expression of gas7 mRNA after addition of cells was investigated by Northern blot analysis by using total RNA prepared from serum starved NIH3T3 cells and at various time after induction into cell cycle by addition of 20% FCS.

The mRNA identified by gas7 probe was 3,1 Kb long and was abundantly expressed in quiescence (figure 2). It has the lowest expression after 6 hours of the addition of serum and then it seems to be induced again even if not at the level of quiescence. The second, lowest peak of expression is 12 hours after the addition of serum.

To normalize each RNA sample loaded on the Northern blot, the same filter was hybridized with the cDNA probe GAPDH, known to remain constant throughout the cell cycle.

The photo shows also the percentage of cells entering S phase for each time.

5. Kinetic of appearance of gas7 mRNA after serum starvation and density-dependent inhibition:

The level of expression of gas7 RNA was analyzed on Northern blots by using total RNA samples extracted at various time after serum deprivation of exponentially growing NIH3T3. NIH3T3 cells were shifted to 0,5% FCS twelve hours after seeding in 10% FCS. Gas7 mRNA seems to be highly induced just after 48 hours of 0,5% FCS (figure 3-top). Normalization of RNA amount was performed with the GAPDH probe on the same Northern blot.

The percentage of cells in the S phase was also measured by analysing BUdR incorporation in cells grown on cover slips for each time point. Less than 15% of the cells remained in the S phase 24 hours after serum starvation. To see the expression of gas7 mRNA in density-dependent growth inhibition, NIH3T3 cells were seeded in 10% FCS and every 2 days the medium was replaced with fresh 10% FCS. Gas7 seems to be slightly induced after 2 days and remains at a steady state level until 8 days when it is up regulated again (figure 3-bottom).

The GAPDH control expression level did not change and DNA synthesis analysis performed on cells grown on cover slips in the same dishes used to extract RNA, showed a significant decrease as early as 2 days after seeding.

6. Pattern of expression of gas7 mRNA after single growth factor addition:

The expression of gas7 mRNA was investigated by Northern blot analysis by using 10 μg of total RNA prepared from serum-starved NIH3T3 cells and at the same time after induction of growth by addition of a single growth factor.

In figure 4-top, the regulation of expression of gas7 after addition of PDGF is shown. The gas7 is slightly down regulated after 6 hours of addition of PDGF and then became to be highly expressed at the 12 hours. The smallest amount of expression is 18 hours from the addition of PDGF but the gas7 mRNA again is induced later at the 22 and 30 hours.

Insulin has a consequence on the expression of gas7 similar to that of PDGF. There is a strong down regulation at 6 hours followed by an increase in the expression of the gene and then a new strong disappearance of the signal. After 30 hours from the addition of Insulin the expression of gas7 is again induced (figure 4-bottom).

FGF is able to down regulate the expression of gas7 slightly already after 1 hour of its addition and very strongly after 6 hours. Then the level of expression goes up again but never reaches the amount of quiescence (figure 5-top).

EGF is able to down regulate completely the expression of gas7. After 6 hours from the addition of the growth factor, the gas7 becomes to be almost undetectable and remain at that level for the whole time course (figure 5-bottom).

Normalization of RNA amounts was performed with the GAPDH probe on the same blot. The percentage of cells in S phase was measured by analizing BUdR incorporation in cells growing on cover slips for each time point for every growth factor.

7. Regulation of expression of gas7:

A nuclear run-on experiment was performed to assess whether transcriptional regulation is responsible for the decreased expression of gas7 mRNA after serum addition in arrested NIH3T3 cells.

Nuclei collected at variuos times after FCS addition to Go NIH3T3 cells synthetized gas7 mRNA at the same level after growth induction.

As controls, the GAPDH transcriptional level seems constant while the γ-actin mRNA was positively regulated during growth induction reaching maximum transcription 30' after FCS addition and decreasing three hours later (photo 9). From this result it's possible to conclude that the expression of gas7 mRNA is regulated at the post-transcriptional level (figure 6).

In order to investigate the mechanisms responsible for the down regulation of gas7 mRNA, two time courses were performed adding to starved cells or Actinomycin D or Cycloheximide together with the serum.

If the Actinomycin D was added together with serum, the expression of gas7 was different. Infact it is induced after three hours of the addition of serum and Actinomycin D and then at six hours it becomes to be down regulated to a level that remains constant and is less than in resting but much more than in the absence of drug (figure 7-top on the left).

The GAPDH control shows, as expected, no differences with or without Actinomycin D.

If Cycloheximide was added together with 20% FCS, the expression of gas7 was very different than in the absence of drug. At six hours it appears to be up regulated and then, from nine to twelve hours it appears to reach a level of expression much lower than in resting phase. At the 18 hours, as in the absence of drug, it is now induced but at a higher level than in resting (figure 7-bottom on the left).

The GAPDH, used as control, shows the usual pattern of expression.

The same experiments with Actinomycin D and Cycloheximide were repeated inducing cell growth just with EGF. The level of the gas7 transcript was always constant suggesting the need of active transcription and translation

to have the EGF-induced down-regulation (figure 7-on the right).

8. Expression of gas7 mRNA in different tissues:

In order to see the presence of gas7 mRNA in vivo, some total RNA were extracted from different tissues of a mouse. From the Northern blot analysis, gas7 results so highly expressed in lung as in resting NIH3T3 cells. It is also highly exprssed in heart, uterus and kidney. All the other tested tissues present a basal level of expression: brain, liver, thymus, spleen, stomach, duodenus and intestine. The lenght of the gas7 mRNA is apparently the same in all the tissues as in the NIH3T3 cells (data not shown).

DISCUSSION

During the last years a lot of data have been accumulated to confirm the existence of two separated networks in the control of cell proliferation.

The positive branch is involved in the exit from quiescence and in the entry of S.

This transition is regulated at different levels and has pleiotropic effects both on the stimulation of S phase and on the inactivation of the quiescence specific metabolic state.

Most of the genes that are present in this pathway are cellular proto-oncogene and their mutated forms are able to over-induce cell growth.

The negative branch acts as an inhibition of G1-S transition working on different moments of that pathway and as activator of the entry in quiescence.

The level of this kind of control has such a dramatic effect on cell proliferation that negative regulators can be targets of mutations involved in the oncogenic process.

For a better comprehension of this second network it is important to see if quiescence specific gene expression occurs and how this process is regulated positively by the negative growth control branch and negatively by the positive one.

In this work I have isolated a new gene, named gas7 (growth arrest specific), whose expression is highly increased in quiescence induced by starvation in NIH3T3.

The minimal amount of gas7 mRNA occurs 6 hours after the induction of growth. This result is coincident with the pattern of expression of all gas genes and other quiescence specific cloned markers. This period is the normal time required to exit from the "out of cycle" state and reach the "in cycle" one. Probably it is the consequence of a different metabolic state that is completely reached just there.

As other gas genes, gas7 mRNA is again induced later at a lower level than quiescence. Particularly it seems to be expressed 12 hours after the addition of serum.

If quiescence is induced by starvation, the gas7 mRNA is at first slightly down regulated and after 48 hours strongly induced.

These data suggest that gas7 is accumulated not during the early step of the process of the entry into quiescence but just when Go is completely stabilized.

Gas7 doesn't seem to be involved in density dependent inhibition process: its expression is just slightly induced and seems to be a very late effect of density-dependent quiescence.

The addition of single growth factor to the cell medium has very different effects in the expression of this gene. PDGF, FGF and insulin provoke a sharp down regulation

of this gene after 6 hours as serum does and a new increase in the expression after 12 hours.

Considering that the percentage of the cell in S phase at that time is completely different for different growth factors, I can hypothized that this down regulation is not related directly to the entry in S phase but to some other metabolic networks that are more related to the "out of cycle" and "in cycle" states.

On the contrary, gas7 is completely differentially regulated by EGF. This growth factor is able to inhibt completely its expression after 6 hours even if a slight reduction of gas7 mRNA appears already after one hour from the addition of the peptide.

This lack of the re-induction of gas7 expression can suggest that different growth factors can not only activate different inducing pathways for the entry in S phase but can act as negative regulators of negative branch in different manners.

Gas7 mRNA expression can be a good model to study the EGF-dependent down regulation.

In the future, the analysis of the gas7 mRNA amounts after treatment of cells with different drugs can help us to dissect the metabolic organization of this negative network.

As most of the gas genes, gas7 seems to be regulated at the post-transcriptional level.

The pattern of its expression in the presence of Actinomycin D and Cycloheximide is very complicated and not very easily interpretable.

I have repeated the same experiment substituting high serum with EGF and I have got a more clear result: the EGF down-regulation of gas7 is mediated by new transcription and new translation of an mRNA and a protein probably involved in the negative control made by the positive growth control branch.

In vivo gas7 is ubiquitously expressed, suggesting that it is probably involved in a fundamental life process.

In conclusion gas7 gene cloning and analysis has confirmed a striking difference between the regulation of genes inducted by serum that are regulated mainly at the transcriptional level and genes inducted by the absence of serum that are regulated mainly at the post-transcriptional level. Moreover gas7 can be a good model to study how the EGF-related positive growth control branch acts on the repression of quiescence genes.

In the future I would like to finish the sequence analysis hoping in a possible homology to some proteins that could help me in the comprehension of gas7's biological function. Moreover I'm going to prepare polyclonal antibodies in order to characterize the gas7 protein. I would like also to study its pattern of expression during embryo development being EGF one of the most important differentiation factors.

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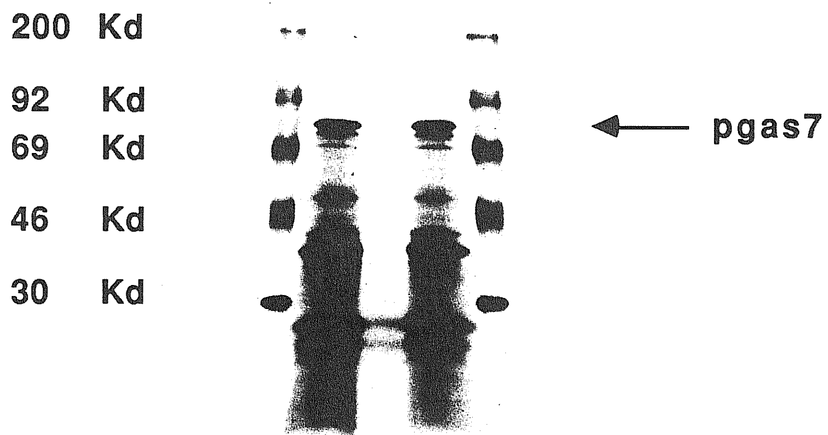
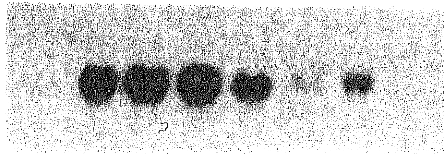
TRANSCRIPTION/TRANSLATION IN VITRO OF gas7



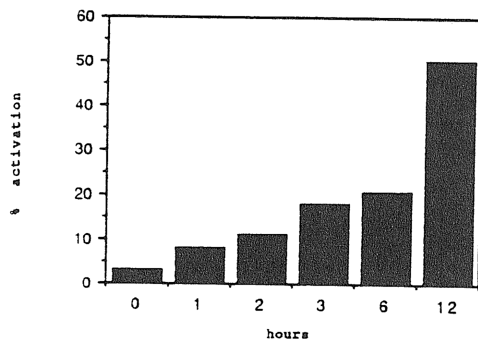
Hours after serum addition

0 1 2 3 6 12



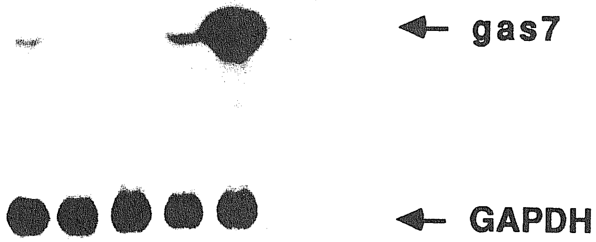
gas7

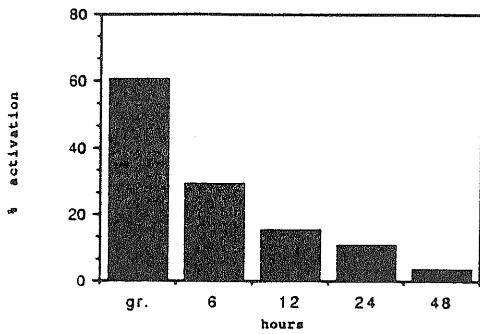
GAPDH



Hours after addition of 0.5% FCS

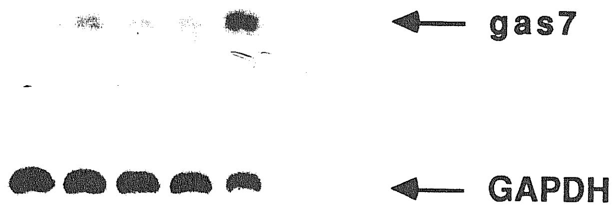
gr. 6 12 24 48

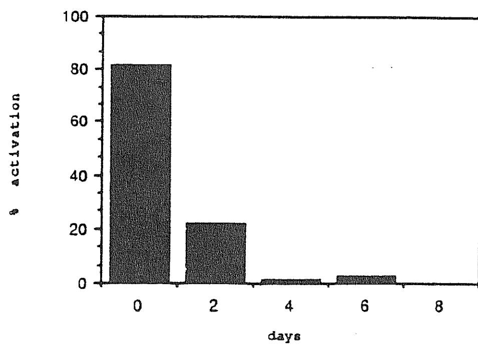




Density Dependent Inibition

gr. 2 4 6 8

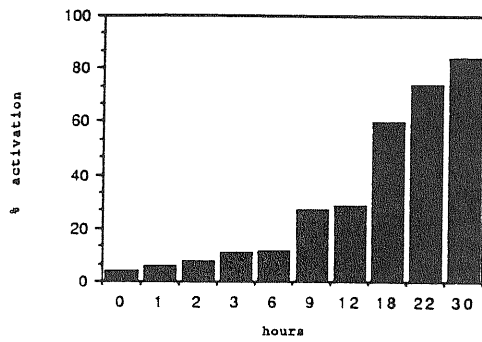




0 1 2 3 6 9 12 18 22 30 PDGF

gas7

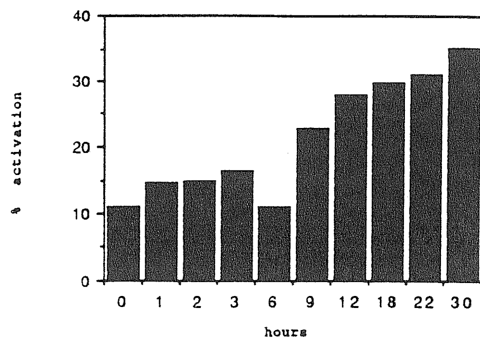
GAPDH



0 1 2 3 6 9 12 18 22 30 Insulin

gas7

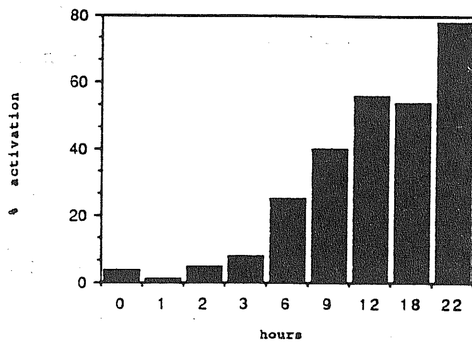
GAPDH



0 1 2 3 6 9 12 18 22 FGF

gas7

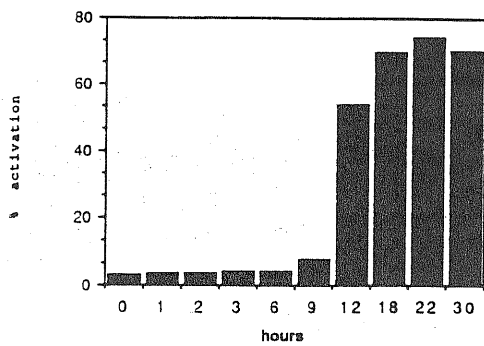
GAPDH



0 1 2 3 6 9 12 18 22 30 EGF

gas7

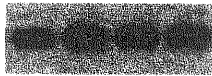
GAPDH



GROWTH INDUCED BY serum

+ Actinomycin D

0 3 6 9



← gas 7 →

← GAPDH →

GROWTH INDUCED BY EGF

+ Actinomycin D

0 1.5 3 6

+ Cycloheximide

0 3 6 9 12 18

← gas7 →

← GAPDH →

+ Cycloheximide

0 1.5 3 4 6

RUN-ON EXPERIMENT