Studies of SV40-transformation and the Loss of Growth Factor Requirements

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ABSTRACT

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I found that SV40 transformation induced the loss of several specific growth factor requirements. In particular, SV40 transformed 3T3 fibroblasts had a significantly reduced growth requirement for insulin. A reduced insulin requirement was also observed in several other transformed cell lines. Dose response studies with insulin and insulin-like growth factors indicated that the mitogenic response to insulin is in all probability mediated by IGF-I receptors, and that the reduced insulin requirement observed in transformed fibroblasts actually reflects the loss of a strong IGF-I requirement. IGF-I is under strict pituitary-growth hormone control *in vivo*, and mediates many if not all of the growth promoting effects of growth hormone. A reduced IGF-I requirement may allow transformed fibroblasts to escape from this major humoral regulatory system.

SV40 transformed cells also displayed a significantly diminished requirement for platelet-derived growth factor (PDGF). The loss of this particular growth factor requirement was found to be closely associated with the loss of density-dependent growth inhibition. Cell lines transformed by temperature sensitive mutants of SV40 exhibited a temperature sensitive loss of the PDGF requirement, indicating that SV40 T-antigen mediates this effect. Results pertaining to the temperature sensitivity of the insulin requirement were inconclusive.

I found that SV40 could directly reduce the insulin requirement of 3T3 cells in a transformation assay based upon the stringent insulin requirement of 3T3 cells for colony formation. Several such insulin-transformants were isolated and characterized. Although all of

these transformants expressed SV40 T-antigen, some of them retained anchorage-dependence and/or a partial PDGF requirement, unlike transformants obtained in the standard density of anchorage assays.

A revertant of SV40 transformed 3T3 cells was found to have regained a very strong dependence upon insulin. This dependence was not overcome by re-transformation with Kirsten Murine Sarcoma virus, although retransformation did obviate both its density-dependent growth inhibition and PDGF requirement. Kirsten transformed 3T3 normally display a greatly reduced insulin requirement, indicating that this particular revertant may have suffered a cellular mutation that prevents transforming viruses from induced the reduced insulin requirement.

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INTRODUCTION

Cellular growth control probably evolved to maximize the survival of a given species. In multicellular organisms, the specific type of growth control exhibited by the cells of a particular tissue is intertwined with the function of that tissue. It is therefore not surprising that different cell types have different ways of regulating their growth. While bacteria and yeast are highly responsive to the nutrient makeup of their environment, mammalian cells are more responsive to growth factors and cell shape and attachment. Within the domain of mammalian cells, there are additional subtypes of growth control. The epidermal cells that line the colon divide fairly rapidly (a 39 hour doubling time) but also have an equal rate of death (Baserga, 1981). Fibroblastic cells do not normally divide in the adult, but remain viable in an intermediate state between the last mitosis and the onset of the next round of DNA replication (Prescott and Mitchison, 1976).

Growth control in bacteria

The manner in which the growth rate of bacteria is highly responsive to the nutrient makeup of their environment is more or less understood. When bacterial cells are shifted from a rich nutrient medium to a poor nutrient medium, the level of amino acids within the cell drops. This leads to the appearance of uncharged tRNA, which somehow directs the ribosome to synthesize ppGpp from GTP. ppGpp in turn binds to RNA polymerase and this leads to a drop in the rate of transcription of rRNA genes as well as other genes (Zubay, 1983). While the cell remains in a viable state, the rate of initiation of DNA synthesis drops sharply (Helmstetter et al., 1968). The rate of increase in cell size is tightly linked to the rate of initiation of DNA synthesis (Jacob et al., 1963). The mechanism that underlies this linkage in unknown, but may involve the attachment of the growing DNA chain to specific membrane components (Hendrickson et al., 1982).

The enzymology involved in the initiation of DNA synthesis is exceedingly complex, on the order of the complexity of the ribosome (Kornberg, 1979). Several bacterial genes are involved in DNA replication (*dnaA*, *dnaB*, etc.). The *rep* gene product, helicase, is an ATPase that separates duplex DNA strands in advance of replication. The "binding protein" stabilizes the single stranded DNA for template formation. Prepriming proteins direct the *dnaB* gene product to the template, which somehow allows the *dnaG* gene product, primase, to transcribe a primer for DNA polymerase (Kornberg, 1979). Although initiation of DNA synthesis at the chromosomal origin of replication has been achieved in an *in vitro* system, not all of the necessary proteins have been purified to homogeneity (Fuller et al., 1981). Probably within 10 years time the mechanism that controls the initiation of chromosomal DNA synthesis will be understood, and hopefully this will shed light on the manner in which cell size is linked to DNA replication.

In certain cases, after a poisoning of bacterial cells with chloramphenicol, chromosomal DNA replication can continue in the absence of protein accumulation (Lark and Lark, 1979). Such aberrant synthesis appears to require the *recA* gene product, which may somehow allow the replication machinery to reinitiate DNA synthesis in the absence of normal regulatory constraints (Lark and Lark, 1979).

Growth control in yeast

Yeast are also highly responsive to the nutrient makeup of their environment (Boehlke and Friesen, 1975). However, another form of growth control is found in yeast. The mating process of **a**-haploid yeast with α-haploid yeast is mediated by diffusible signals, i.e. hormones. The **a**-haploid yeast can secrete **a**-factor, which causes α-yeast to arrest in G1 and synthesize cell surface agglutinins (Thorner, 1982). The α-yeast can secrete α-factor, which similarly affects **a**-cells. Both cell types become arrested in G1, and subsequently fuse.

α-factor is known to inhibit the activity of membrane bound adenyl cyclase (Thorner, 1982). Further evidence that α-factor acts by lowering cAMP levels comes from the existence of a mutation *cyr1*. *cyr1* mutants require exogenous cAMP for growth, and arrest in G1 in its absence (Thorner, 1982). Revertants of *cyr1* mutants were found that appear to have suffered second site mutations effecting the cAMP binding regulatory subunit of cAMP dependent protein kinase (Thorner, 1982). The picture emerging from such studies is that cAMP is required for the growth of yeast cells, and that this effect, like all other cAMP effects found thus far in eukaryotes, is mediated by cAMP dependent protein kinase. Decreasing cAMP concentrations leads to G1 growth arrest.

Growth control in the developing organism

The adult human body contains on the order of 10^{14} cells, all derived from a single cell, the fertilized egg (Prescott, 1978). Most of this cell division, in terms of absolute number of cells, occurs after birth. However, it is instructive to note that the most rapid rate of growth occurs immediately after conception. The cleavage stage divisions are very rapid and there is no lag between mitosis and DNA synthesis, i.e. there is no G1 period (Prescott, 1978). After the cleavage stage is over, a G1 period is introduced, but the cells continue to divide rapidly. The growth rate slows down as the embryonic cells begin to differentiate, and some differentiated cells within the embryo begin to grow more slowly than others (Spratt, 1971). Within a single tissue, such as skeletal tissue, some of the cells cease dividing, and the actively growing cells become localized in so-called growth centers (Spratt, 1971). Such selective control of cell proliferation within a single tissue is likely to be intimately involved with the formation of specific shapes, such as the wing bud of chicks (Cooke and Summerbell, 1980). The formation of specific shapes, such as the fingers, is also accompanied by

cell death. Cells in the developing hand will die in order to form the spaces in between the fingers (Spratt, 1971).

Thus, developmental growth control is achieved by the selective differentiation of embryonic cells into morphologically distinct cells that proliferate at different rates, by the decrease of the percentage of actively growing cells and their localization into growth centers, and by cell death.

How is the rapid growth rate of embryonic cells achieved? The fertilized egg is very large, hence the G1 minus cleavage divisions are not impeded by the need to accumulate cellular components. The rapid rate of growth following cleavage divisions is accompanied by a high level of expression of the p53 protein (Chandrasekaran et al., 1981). The p53 protein is a nuclear protein whose activity is required for serum stimulation of DNA synthesis in Swiss 3T3 cells (Mercer et al., 1982). The p53 protein may function in the regulation of the initiation of DNA synthesis, and the rapid growth rate of embryonic cells may result from the high level of expression of this protein.

Several reports have documented the production of growth factors by embryos. Mouse embryos are rich sources of so called 'transforming growth factors', which are peptides that can induce certain normal cells to grow in soft agar (Moses et al., 1981). Additionally, the level of the insulin like growth factor MSA is 20-100 fold higher in fetal rat serum than it is in adult rat serum (Moses et al., 1980). This insulin like growth factor is distinct from the adult form of insulin like growth factors, which do not reach normal levels in humans until age 6 (Sara et al., 1981). Radioreceptor assays that employ fetal membranes have been used to demonstrate a correlation between birth size and the level of the so-called embryonic insulin like growth factor in humans (Sara et al., 1981). In one case, a high level of embryonic insulin like growth factor was found in an anencephalic fetus (Sara et al., 1981). Normally, insulin like growth factor levels are dependent upon pituitary function.

F9 embryonal carcinoma cells resemble the inner cell mass of the early mouse embryo (Strickland and Mahdavi, 1978). These cells can be induced to differentiate into parietal endoderm by the addition of retinoic acid to the culture medium (Strickland and Mahdavi, 1978). Retinoic acid is known to inhibit the binding and function of 'transforming growth factors' (Todaro et al., 1978). Part of the differentiation process may involve the cessation of synthesis of or response to embryonic growth factors.

Endocrine control of growth in adults

The major regulatory system controlling growth in later human life is the pituitary growth hormone system (Daughaday et al., 1975). Low levels of growth hormone results in dwarfism, and high levels of growth hormone results in gigantism. If high levels of growth hormone persist after bone ossification is complete, the result is acromegaly, a condition of uncontrolled proliferation of bone cells and fibroblasts (Daughaday et al., 1975).

Growth hormone does not appear to affect the growth of cells directly. However, serum from hypophysectomized rats injected with growth hormone can stimulate cell division directly. This observation led Daughaday to propose the somatomedin hypothesis (Daughaday et al., 1975), which maintains that the growth promoting effect of growth hormone is mediated by a secondary hormone. A protein that fulfills this predicted role has been found. It has been termed somatomedin C by one laboratory (Marshall et al., 1974) and insulin like growth factor I (IGF-I) by another laboratory (Rinderknecht and Humbel, 1976). Only IGF-I has been purified to complete homogeneity, and it has been found to be under strict growth hormone control. Injection of IGF-I into hypophysectomized rats can result in significant longitudinal growth, lending credence to the somatomedin hypothesis. IGF-I is structurally similar to insulin (Blundell and Humbel, 1980), but is some 100 times more potent in stimulating the growth of fibroblasts (King and Kahn, 1981).

IGF-II, another growth factor which is structurally similar to insulin, is not under growth hormone control (Czech, 1982). IGF-II appears to be closely related to MSA, the putative embryonic form of somatomedin. IGF-I, IGF-II, insulin, nerve growth factor, and relaxin are all members of the insulin family of hormones, and they probably all evolved from a common ancestral gene (Blundell and Humbel, 1980). All members of this family have distinct cell surface receptors, and in human fibroblasts

and Swiss 3T3 cells, the IGF-I, IGF-II, and insulin receptors are expressed (Czech, 1982).

Other growth factors are likely to play a role in growth control in adults. As mentioned previously, some tissues have rapidly dividing cells. This is particularly true of the epidermal cells that line the digestive tract. Epidermal growth factor/urogastrone is a growth factor that stimulates epidermal growth *in vivo* (Carpenter and Cohen, 1979), and may play a role in stimulating the rapid proliferation of the epidermal cells that line the gut. While low levels of this growth factor (1 ng/ml) are found in plasma, high levels (300-1000 ng/ml) are found in saliva, urine, and breast milk (Carpenter and Cohen, 1979). EGF has not been shown to be mitogenic for fibroblasts *in vivo*, although it can stimulate DNA synthesis of fibroblasts in culture (Carpenter and Cohen, 1979).

During the wounding process, platelets are stimulated to release the contents of their vesicles and granules, which contain serotonin, calcium, and platelet derived growth factor (PDGF), among other things (Ross and Vogel, 1978). PDGF is a chemoattractant for both smooth muscle cells and fibroblasts (Grotendorst et al., 1981). In addition, it is a potent mitogen specific for these cell types. PDGF receptors are not found on endothelial or epithelial cells (Heldin et al., 1981b). PDGF can stimulate human fibroblasts to secrete IGF-I, which probably augments the mitogenic response to PDGF (Clemmons and Van Wyk, 1981; Clemmons et al., 1981). PDGF has been referred to as the wounding hormone because of its proposed role in inducing the migration of fibroblasts and stimulating them to divide (Ross and Vogel, 1978). Interestingly, purified PDGF, unlike EGF or insulin, is able to prevent the differentiation of 3T3 L1 fibroblasts into adipocytes (Hayashi et al., 1981). Thus, PDGF may also be viewed as a hormone that maintains the differentiated state of the fibroblast for wound related functions, e.g. collagen secretion.

Several other growth factors are known to exist; T cell growth factor (Leonard et al., 1982), erythropoeitin (Harrison, 1976), renal growth factor (Nomura et al., 1982), and skeletal growth factor (Maugh, 1982). All of these growth factors show at least some target tissue specificity.

How do growth factors stimulate cells to divide? The answer to this question is not known. A number of years ago, there was an inordinate effort on the part of many scientists towards proving that growth factors induce cell division by increasing nutrient uptake, perhaps because of the close relationship between nutrient availability and growth rate in lower organisms (Holley, 1975). However, critical experiments failed to substantiate this hypothesis (Dubrow et al., 1978; Thrash and Cunningham, 1974). In fact, nutrient uptake was shown to be cleanly dissociated from growth control (Weber and Friis, 1979). In retrospect, it seems odd for so many scientists to have assumed that an organism that requires the constant functioning of quiescent cells (e.g. neurons, which use a lot of nutrients, but do not divide), would employ nutrient availability as a means of growth control.

A more fruitful line of investigation appears to be the involvement of the cytoskeleton in growth factor stimulation of mitogenesis. Cytochalasin can disrupt the actin cytoskeleton of 3T3 fibroblasts and can act to prevent serum stimulation of DNA synthesis (Maness and Walsh, 1982). Colchicine and other microtubule disrupting agents can inhibit the serum stimulation of subconfluent cells (McClain and Edelman, 1980). Drugs that block the activity of calmodulin, a calcium binding regulatory protein that interacts with the cytoskeleton, also inhibit serum stimulation of growth (Chafouleas et al., 1982). Moreover, growth factors themselves can induce dramatic changes in the cytoskeleton. For example, EGF can induce the disorganization of the typical actin and alpha actinin structures of cultured cells (Schlessinger and Geiger, 1981).

Ultimately, growth factors may act by turning on specific cellular genes. Support for this hypothesis comes from studies with drugs that interfere with RNA pol II transcription. A cell line that has a temperature sensitive defect in RNA pol II does not enter S phase at the non permissive temperature (Baserga, 1981). Recently it has been demonstrated that the mitogenic signal induced by PDGF is RNA, presumably mRNA (Smith and Stiles, 1981). cDNA clones representing mRNA preferentially synthesized in response to PDGF have been isolated.

Cancer is a loss of growth control

Colon carcinoma is not a rapidly dividing type of tumor. Indeed, its growth rate is slower than that of normal colon epithelial cells (Baserga, 1981). However, normal colon epithelial cells have a balanced rate of death, whereas colon carcinoma cells do not. Thus, the loss of growth control in colon carcinoma cells can be thought of as the inability to terminally differentiate.

It is of interest that epithelial cells will undergo rapid terminal differentiation when they are placed in methocel suspension culture (Rheinwald and Beckett, 1980). Perhaps the loss of attachment to a solid substrate or the inability to spread out to a flat shape is part of the normal process that signals epithelial cells to terminally differentiate (Pollack, 1980). In contrast, carcinoma cells continue to divide in methocel suspension culture (Rheinwald and Beckett, 1980).

Fibroblasts are loosely packed within a collagen meshwork that underlies the epidermal layer (Gabbiani et al., 1972). Normally, fibroblasts do not divide in the adult animal, except in response to injury or in pathological conditions such as acromegaly (Daughaday et al., 1975). The inappropriate growth of fibrosarcomas can be viewed as a failure of these cells to enter a resting state of no division.

SV40 transformation of fibroblasts

To date, the most intensely studied tumorigenic process has been the conversion of a normal fibroblast into a tumorigenic fibroblast by the small DNA tumor virus SV40. The SV40 DNA needs to become stably associated with host cell DNA in order for this conversion to occur (Tooze, 1980). In the case of SV40, this stable association requires the integration of SV40 DNA into host chromosomal DNA (Tooze, 1980). A related tumor virus, papilloma virus, can become stably associated outside the host chromosome as an episomal element (LaPorta and Taichman, 1982).

It does not appear as though any special site of integration is required for tumorigenic transformation of fibroblasts by SV40 (Tooze, 1980). A single inserted copy of the SV40 early region appears to be necessary and sufficient for the transformation of rat fibroblasts (Steinberg et al., 1978). The early region of SV40 codes for two polypeptides, the 17K tumor antigen (t-antigen) and the 96K tumor antigen (T-antigen) (Prives et al., 1977). The 17K t-antigen is not required for tumorigenicity, although its presence enables tumors to grow more rapidly (Tooze, 1980). The 96K T-antigen appears to be an absolute requirement for tumorigenicity (Tooze, 1980).

T-antigen is required for viral replication during the lytic cycle of the virus. Since SV40 encodes only 5 or 6 genes, 3 of them coding for structural capsid proteins, its replication strategy calls for the induction of host cell machinery involved in DNA synthesis. Indeed, infection of permissive cells with SV40 will induce one round of host cell chromosomal DNA synthesis. After that, SV40 DNA will continue to replicate. T-antigen binds to the SV40 origin of replication and is required for the initiation of new rounds of viral DNA synthesis (Scheller et al., 1982; Tegtmeyer, 1972; Tjian, 1978).

Infection of cells with SV40, but not with adenovirus, not only induces host cell DNA synthesis, but also causes the cells to increase their ribosomal RNA content (Baserga, 1981). Adenovirus infection stimulates DNA synthesis without concomitant

stimulation of rRNA synthesis (Baserga, 1981). SV40 is therefore similar to serum growth factors, which also induce balanced growth (Baserga, 1981). By microinjection of various SV40 DNA fragments into cells, it was shown that the induction of DNA synthesis can be dissociated from the increase in rRNA synthesis (Baserga, 1981). Apparently, the ability of T-antigen to carry out each of these two functions resides in different domains of the molecule.

In transformed cells, integrated SV40 DNA can undergo amplification and rearrangement (Blanck et al., 1983). Amplification appears to be regulated by T-antigen, as cell lines transformed with temperature sensitive SV40 mutants are temperature sensitive for SV40 DNA amplification (Lavi, 1981). Preliminary work from our laboratory suggests that a functional SV40 origin of replication is required for SV40 DNA rearrangement. Most likely, T-antigen is binding to the SV40 origin of replication and inducing unscheduled DNA replication. In at least some cases, the resulting rearrangement of SV40 DNA is associated with the production of super-sized T-antigens. These super sized T-antigens may be more potent stimulators of the transformed phenotype than normal sized T-antigen (Chen et al., 1981; Clayton et al., 1982).

T-antigen may influence the transcription of cellular genes. Several cDNA clones have been isolated that represent mRNA preferentially synthesized at the permissive temperature in cells transformed by temperature sensitive mutants of SV40 (Schutzbank et al., 1982). Whether this preferential synthesis of certain cellular genes is involved in cellular transformation remains to be determined.

The ability to grow in methocel or soft agar suspension is induced in some but not all SV40 transformed cells (Risser and Pollack, 1974). Normal fibroblasts will enter a G1 growth arrest state when placed into suspension. The ability to grow without anchorage is a necessary prerequisite for tumorigenic growth (Kahn and Shin, 1979). This observation, originally discovered with SV40 transformed cells, has been

confirmed for a wide variety of transforming agents, and for both fibroblastic and epithelial cells (Kahn and Shin, 1979). Closely associated with the ability to grow in methocel is a dramatic change in the In most cases, studies with temperature actin cytoskeletal structure (Pollack et al., 1975). Temperature-sensitive mutants that effect T-antigen indicate that a functional T-antigen is required for both anchorage independent growth and an altered cytoskeleton (Tooze, 1980). The 17K T-antigen may have an enhancing role in these changes as well (Rubin et al., 1982; Steinberg and Pollack, 1979). Other transforming viruses can induce disruption of the cytoskeleton (Sefton et al., 1981). Certain components of the cytoskeleton, e.g. vinculin, are direct targets for the enzymatic function of transforming proteins (Sefton et al., 1981).

Anchorage independent SV40 transformed cells produce 'transforming growth factors' that stimulate certain normal cells to grow in soft agar (Kaplan et al., 1981). Several SV40 transformants have been found to secrete a PDGF like molecule (A. Vogel, personal communication).

Although much has been discovered about SV40 transformation, the exact mechanism by which SV40 T-antigen causes tumorigenic growth is still unknown.

CHAPTER 1

DEVELOPMENT OF SERUM FREE MEDIUM FOR MOUSE FIBROBLASTS

Abstract

Starting with the partially defined medium developed by Sato's laboratory for the serum free growth of Swiss 3T3, a totally defined medium was developed that supports the growth of Swiss 3T3 cells. For cells plated at a density of 10 cells/mm^2 or higher, this medium stimulates 3T3 to grow at a rate comparable to that obtained with 10% serum. This totally defined medium employs fibronectin coated dishes, insulin, platelet-derived growth factor, epidermal growth factor, transferrin, and the rich basal medium MCDB 402. 3T3 cells can form colonies in this medium, albeit the growth obtained is not as extensive as that obtained with 10% serum.

Introduction

For years, cell culturists attempted to grow various tumors in culture. In most cases, normal fibroblasts would outgrow the original tumor explant. Gordon Sato and his colleagues developed a method that allowed several tumors to become successfully established as cultured cell lines. This method involved the repeated serial passage of the tumor explant in culture followed by passage of the surviving cells *in vivo*. Fibroblasts were selected against during the *in vivo* passages, and tumor cells that could survive and grow in culture were selected for during the *in vitro* passages (Buonassisi et al., 1962). Cell lines derived from a pituitary tumor, an adrenal tumor, and an ovarian tumor were established in this manner (Clark et al., 1972). In some cases, these tumor cell lines retained some of the differentiated functions that were present in the original tumor.

Previous to this accomplishment, Jacob Furth had demonstrated that breaking the feedback loop that exists between the ovary and the pituitary could induce ovarian tumors. Ovarian granulosa cells are stimulated to divide by gonadotropins released by the pituitary. Ovarian cells in turn secrete estrogen and progesterone that turn off the secretion of gonadotropins by the pituitary. If the ovaries are transplanted to the spleen, all of the blood that passes through the ovaries will pass through the liver before reaching the main circulation system of the animal. The liver inactivates estrogens, thus preventing the pituitary from receiving the signal to stop secreting gonadotropins. Gonadotropins continue to be produced, and the ovarian cells continue to proliferate. Eventually, the continued proliferation of ovarian cells will yield an ovarian cell tumor (Furth and Sobel, 1947). These tumors remain hormone dependent; they will not grow in hypophysectomized animals (Furth, 1969).

Ovarian tumor cell lines grown in culture did not show growth response upon the addition of luteinizing hormone to the growth medium. Gordon Sato believed that this result was brought about by culture conditions. He reasoned that the necessity of growing cells with 10% serum prevented one from observing a growth response to this hormone and possibly other hormones as well. To test this hypothesis, Sato treated serum with carboxy-methyl cellulose that removed basic peptides, including luteinizing hormone, from the serum. When serum treated in this manner was used to culture ovarian tumor cells and luteinizing hormone was added to the culture medium, a growth response to luteinizing hormone was detected (Clark et al., 1972). This discovery led Sato and colleagues to attempt to totally replace serum with hormones. The first cell line successfully grown with hormones in lieu of serum was the rat pituitary cell line GH3 (Hayashi and Sato, 1976). Ironically, the discovery of a growth response to luteinizing hormone that spurred the development of a hormonally defined medium was later found out to be caused by an impurity present in the hormone preparation employed by Sato and his colleagues (Gospodarowicz and Moran, 1976)

Several growth factors and other proteins have been crucial, to the development of serum free media. Virtually all cell lines require insulin for optimal growth (Barnes and Sato, 1980). In some cases, this requirement has been shown to be due to the ability of insulin to mimic insulin-like growth factors. In other cases, such as the insulin growth response of a rat hematoma cell line (Massague et al., 1982), the response to insulin occurs at such low physiological concentrations that it is likely that the high affinity insulin receptor is mediating this effect. Fibronectin, a large glycoprotein present in plasma and on the surface of many cells, has been important in replacing the serum-coating of dishes that is often otherwise necessary for serum free growth (Barnes and Sato, 1980). Transferrin, the iron transport protein of plasma, is often included in serum-free media, as many cells have specific cell-surface receptors for transferrin (Barnes and Sato, 1980). The requirement for transferrin is obviated when freshly prepared ferrous sulfate is added to the medium (Walthall and Ham, 1981).

Richard Ham has established that trace elements, like zinc and selenium, are necessary components of defined media (Ham, 1965). In all cases, serum free growth is optimized only when trace elements are added (Barnes and Sato, 1980). Ham's work has been crucial to the development of serum free media for normal, non-tumorigenic cells. Ham's laboratory adopted a different approach from Sato in their attempt to replace serum by balancing the components of the nutrient medium. They optimized each component of the basal medium for clonal growth in low serum. Utilizing this approach, Ham found that he could completely replace serum for the growth of CHO cells (Ham, 1965). Embryonic human fibroblasts, however, could not be successfully grown at clonal densities without low amounts of serum (Walthall and Ham, 1981). Ham hypothesized that the residual amount of serum required by these cells served to regulate the balance of nutrients, and he believed that this requirement would eventually be obviated by complete nutrient optimization. However, his laboratory has now

adopted the approach of replacing the residual serum requirement with purified growth factors (Walthall and Ham, 1981).

The development of serum free medium for the growth of normal cells has allowed investigators to begin an examination of the loss of growth factor requirements following neoplastic transformation. In an early study, normal hamster fibroblast cell lines (BHK-21 and CHEF-18) were initially plated in 10% FCS, then switched to serum free medium containing EGF, FGF, insulin, and transferrin (Cherington et al., 1979). CHEF-18 cells did not respond to FGF, but responded to both EGF and insulin. Transformed derivatives of CHEF-18 lost or had significantly diminished requirements for EGF (Cherington et al., 1979). It has been shown that a reduced EGF requirement may also be a feature of frank carcinomas of the colon. In one study, cells derived from preneoplastic polyps still showed an EGF growth response, whereas those derived from frank carcinomas did not (Friedman et al., 1981).

In the study with hamster fibroblasts mentioned above, the authors concluded that the transformed cell lines retained their parental cell line's insulin requirement (Cherington et al., 1979). However, this conclusion was based upon the observation that the transformed cells still responded to insulin. Their insulin response was not quantitatively compared with the insulin response of the parental cell line. A later publication revealed that the insulin requirement of these transformed cell lines, in terms of relative doublings, was reduced (.79 relative doublings for the tumor-derived cell line, and .39 relative doublings for the parental cell line) (Pardee et al., 1981).

Gordon Sato's laboratory has also reported a quantitative reduction in the growth requirement for insulin in certain transformed cell lines. The measure of the insulin requirement in this case was the concentration that promoted optimal growth. Both SV40-transformed Balb/3T3 cells (Rockwell et al., 1980) and Moloney Murine Sarcoma Virus transformed MDCK epithelial cells (Taub et al., 1981) displayed a 10-fold reduction in the insulin concentration required for optimal growth, compared to

their normal parental cells. In another series of experiments, Sato and his colleagues examined the growth factor requirements of SV40-transformed Balb/3T3 cells for FGF, HGF, and "gimmel" factor, a crude salivary gland extract of rat submaxillary glands (Serrero et al., 1979). Although the requirements were not directly compared-in the same study, nor under the same conditions, Sato and colleagues concluded that SV40-transformed Balb/3T3 had lost the requirements for FGF, EGF, and "gimmel" factor. These growth factors were mitogenic for Balb/3T3 grown with a serum coat in place of the fibronectin coating used in the study of SV40-transformed 3T3 (Rockwell et al., 1980). Other laboratories have demonstrated a modest EGF requirement in SV40--transformed 3T3 cells, indicating that a total loss of the EGF requirement is not a universal feature following SV40 transformation (Carpenter and Cohen, 1979; Young and Dean, 1980).

Topp and colleagues used a colony formation assay to compare the growth requirements of SV40-transformed rat embryo cell lines with the requirements of the normal parental cell line (McClure et al., 1981). The medium employed contained insulin, transferrin, hydrocortisone, high-density lipoprotein (HDL), EGF, vasopressin, and fibronectin. Transformed lines in some cases displayed a reduced requirement for HDL, and in all cases, displayed a reduced requirement for fibronectin. Individual omission of any one of the polypeptide growth factors, EGF, vasopressin, or insulin, did not significantly decrease the growth of the parental cell line. Dual omission of EGF and vasopressin diminished the growth of the parental cell line more than it did the growth of the SV40-transformants (McClure et al., 1981). The lack of a strong insulin requirement and the low insulin concentration required for optimal growth by the normal rat fibroblast cell line observed in these experiments is at odds with the results obtained in our laboratory and in Richard Ham's laboratory. It is possible that the 80 μg/ml HDL used in the experiments conducted by Sato, Topp and colleagues contained significant insulin-like growth factor activity. HDL is separated from other plasma

components by density centrifugation without any subsequent chromatographic separation, so it is not unlikely that some contaminants were present in the HDL preparation. A 1 in 10,000 contamination of IGF-I would cause a significant growth response (Chapter 2).

I have gone into considerable detail in the last few pages to bring into focus some of the work that is seemingly at odds with the major conclusion of my thesis work, which is that transformed fibroblasts have a significantly reduced growth requirement for insulin.

Materials and Methods

Preparation of Water

Most laboratories using serum free medium use glass distilled water. According to Gordon Sato, this water must be free of pyrogens, a common contaminant of distilled water (Bottenstein et al., 1979). The method of preparation that I employ, a slightly modified version of Sato's protocol, is as follows. House distilled water is deionized by passage through a Barnstead mixed-bed ion exchanger. This deionized, distilled water is mixed sequentially with 40 g/ 4 L of potassium permanganate and 4 ml of concentrated sulfuric acid. The addition of these chemicals provides strong oxidizing conditions in order to break down organic contaminants like pyrogens. This mixture is distilled in a 2 liter round bottom flask connected up to a Frederick condenser. Glass boiling beads are required to prevent bumping. The distillate is collected until approximately 20% of the original mixture remains in the boiling flask. Initially, the distillate is clear. However, when the azeotrope is reached, the distillate starts to become pink. The distillation process is halted shortly thereafter. After use, the boiling flask is rinsed several times with distilled water, before the process is repeated. Periodically, the boiling flask must be cleansed of the scaly buildup of dark brown residue. The only solvent that will

dissolve this buildup is concentrated HCl. This acid washing process is carried out in a well-ventilated hood.

The distillate is added to a second round bottom-boiling flask of 4 liter capacity. This flask should contain a lot of boiling beads, otherwise, severe bumping occurs. This flask is connected to two Frederick condensers via a trap that prevents water droplets that rise with the vapor from entering the condensers. The condensers are vented on the top to allow the escape of volatile gases. This second flask is also cleansed periodically with HCl.

The final distillate is not kept at room temperature for lengthy periods. It is wrapped with parafilm and stored at 4^{o} C, in an attempt to minimize equilibration with the atmosphere.

Preparation of Glassware

All glassware used in my experiments is treated as follows. The glassware is soaked in 4% HC1 (vol/vol) overnight. It is then soaked in 4% NaOH (wt/vol) for no longer than 10 minutes; longer periods cause the NaOH to etch the glass. The glassware is then rinsed vigorously with distilled water for at least ten minutes. Any residue of NaOH feels soapy to the touch and indicates that the rinsing process is not complete. Once the glassware is thoroughly rinsed, it is rapidly air-dried. Bottles are topped with aluminum foil, and pipets are put into metal canisters. The glassware in then sterilized for four hours or longer in a sterilizing oven. Bottle tops are wrapped in aluminum foil and autoclaved. If a bottle has been used solely to store PBS or medium, it is merely rinsed once with distilled water and resterilized. Any glassware that has come into contact with any protein is recycled through the acid and base wash described above before sterilization. No detergents are ever used.

Preparation of Stock Solutions

Stock solutions for the medium MCDB 402 are made up every six months. This medium, which is derived from DME, was developed in Richard Ham's laboratory for the clonal growth of Swiss 3T3 cells. Stock solutions lA, 1B, and 1C contain most of the amino acids (Sigma). They are made up in 900 ml quantities in a 1000 ml graduated cylinder, and are then stored at -20^{o} C in 1 liter polypropylene bottles that have been acid/base washed. Stock solution 1D is not used; instead, glutamine is added directly during the preparation of the medium, and asparagine is made up in 100 ml quantities as a 100X solution, stored in 50 ml polypropylene tubes at -20^{o} C, and is thawed and added to the medium during preparation.

All other stock solutions are made up in 100 ml quantities and stored in 50 ml tubes. Those vitamins that require dessicated storage (biotin, choline chloride) are periodically reordered (Sigma) if it has become apparent that they have absorbed too much moisture. Putrescine, lipoic acid, and biotin are made up as separate, concentrated stocks before being combined into stock solution 11. Putrescine is made up as a 1 mM solution in water. Lipoic acid is made up as a 1 mM solution in 95% ethanol. Biotin is made up as a 1.5 mM solution in 95% ethanol. The final concentration of biotin in the medium is 100 ng/ml. Ham observed no added effect when biotin was added to the medium, and therefore included it at low concentrations in MCDB 402. Other investigators, working with serum free growth of SV40 transformed 3T3 cells (Young et al., 1979), found that 100 ng/ml optimized the growth of these cells. Likewise, Ham did not see any effect on cell growth when Vitamin B12 was added to the medium, and it too was included in MCDB 402 at low concentrations. Other investigators noted a positive effect of 3 x 10^{-7} M vitamin B12 for the, serum free growth of mouse 3T6 fibroblasts (Mierzejewski and Rozengurt, 1976). The final concentration of Vitamin B12 I use in the medium is also 3 x 10^{-7} M. Since I am employing an entirely serum free medium, I have chosen to use both biotin and vitamin B12 in my medium at higher concentrations than those prescribed by Ham.

The trace metal solutions are made up from concentrated (1 mM) stock solutions that are acidified with 1 drop of HC1 per 100 ml. Ferrous sulfate is deleted from stock 9; instead, it is made up and added separately each time the medium is made. Sodium silicate is added at a 10-fold lower concentration than is prescribed in the formula for MCDB 402 (Shipley and Ham, 1981). Other media by Ham do not employ the high concentration given in the recipe for MCDB 402, and since no mention was made in Ham's paper as to why such a high concentration was employed, I decided to use it at the lower concentration. The trace metal solutions and stocks 9 and 10 are stored in 50 ml tubes at 4^{o} C.

Preparation of MCDB 402

Approximately 600 ml of water is added to a specially cleansed 1 liter cylinder. Five millimoles each of HEPES and Sodium HEPES (Sigma) are then added, and the solution is stirred. Stocks lA, 1B, and 1C are added at 50 ml each, using a specially cleansed 100 ml cylinder. Stock solutions 2 through 6 and 8 through 16 are then added with polystryene pipets. The solid components, including glutamine, are added shortly thereafter. 50 mg of gentamicin sulfate (Sigma) is added. Calcium chloride (Stock 7) is added next, with rapid stirring. The volume is brought up to 1000 ml, and 3.0 g of sodium bicarbonate is added. The medium is gassed with CO_2 until it turns orange in color. The final pH in the incubator will be 7.3. Nalgene filters are washed once with 90^{o} C distilled H20 to remove the detergents used to preserve the membrane. Next, a small amount (10 ml) of the medium is used to rinse the filter. The medium is then sterilized by passage through the Nalgene filter. The sterilized medium is stored at 4^{o} C in glass bottles. The NaCl concentration in the medium is reduced to 4.6 g/L from the concentration recommended by Ham. The reasons for this reduction are discussed in the Results section.

Preparation of Poly-D-lysine

50 mg of poly-D-lysine (MW 30,000 Sigma) are dissolved in 50 ml water in a 50 ml polypropylene tube. This solution is then sterilized in a pre-washed Nalgene filter, and the volume is brought up to 500 ml with sterilized water. Two glass bottles, one 100 ml, the other 500 ml, are set aside especially for poly-D-lysine. Since poly-D-lysine sticks to the glass bottle and may subsequently leach out into whatever solution in contained therein, these bottles are never used to store anything except poly-D-lysine.

Preparation of PBS

PBS is made up in glass-distilled water, and filter sterilized as described above. A sterile stock solution of 0.1 M EDTA is used to make up 100 ml amounts of 0.5 mM EDTA/PBS.

Preparation of Fibronectin

Human plasma is obtained from the blood bank at St. Luke's Hospital. One unit of fresh plasma is passed through a 20 ml gelatin-affinity column (Affi-Gel, Bio-Rad) running under gravity and at no particular flow rate. The column is then washed with 1 liter of PBS and bound fibronectin is eluted with 1 M NaBr, 10 mM sodium phosphate, pH 5.5. Approximately 50 ml of eluate is collected. The eluate is dialyzed against 100 times its own volume in PBS (lab stock) for two days, with two changes of the dialysis buffer. The fibronectin is then sterilized by passage through a Nalgene filter. The fibronectin is stored at -20^{o} C in 50 ml polypropylene tubes. Fibronectin sticks tenaciously to glass and plastic, and approximately 50% of it is lost during the sterile filtration process. Therefore, the concentration of fibronectin must be determined after filtration. A sterile polypropylene pipet tip is used to draw up some of the solution that is then discharged. Using the same tip, a second aliquot of the solution is drawn up. This is used to determine the absorbance of the fibronectin solution at 280 nm on a Zeiss PM

6 spectrophotometer. Fibronectin has an extinction coefficient of E280 = 1.3 mg/ml (Engvall and Ruoslahti, 1977). A small aliquot of unfiltered fibronectin is used to check the purity of the preparation. It is analyzed on a 5-15% polyacrylamide gradient gel, using commercial fibronectin (Sigma) as a standard (Figure 1-1).

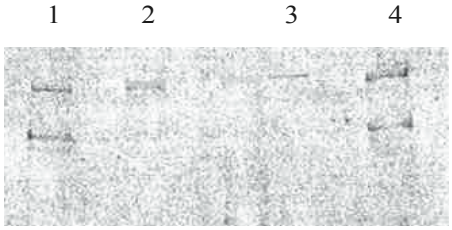

Figure 1-1. Fibronectin was analyzed on a 5-15% polyacrylamide gel. Lanes 1 and 4 were loaded with collagen to serve as molecular weight markers (200 and 100 kD; fibronectin is 220K kD). Lane 2 is prepared fibronectin, Lane 3 is commercial fibronectin (Sigma).

Preparation of PDGF

The PDGF used in these experiments was purified according to the procedure described by Heldin (Heldin et al., 1981a). 200 units of outdated human platelets were obtained from the New York Blood Center. I carefully emptied the contents of these packages into a large flask in a Baker hood. I wore gloves and took care not to come into direct contact with the platelets. All materials that cane into contact with the platelets were autoclaved. These precautions were taken because there is a 0.5% chance that a unit of platelets contains a viable Hepatitis B virus.

Platelets were isolated from the plasma in which they are stored by centrifugation in large DuPont centrifuge bottles using a GSA rotor at 4,500 rpm for 10 minutes. The supernatant was removed, and the packed platelets were washed once with PBS, and frozen at -20 0 C. They were then thawed, and suspended in 800 ml distilled

water. To promote their disintegration, the platelets were frozen and thawed five times. Then, 400 ml of 1 M NaCl, 10 mM sodium phosphate, pH 7.4, was added. The suspension was centrifuged at 9500 rpm in the GSA rotor for 4 hours. The supernatant was removed and dialyzed for 3 days against 8 liters of 80 mM NaCl, 10 mM sodium phosphate, pH 7.4, at 4 0C. The dialysis buffer was changed each day. When the dialysis was completed, 20 g of dry CM-Sephadex (high capacity, Sigma) was added and the slurry was shaken on a rotary shaker overnight at 40C.

The slurry was then poured into a very large column (on loan from S. Beychok). The operating pressure was not allowed to exceed the length of the packed column bed (about 2 feet). The column was washed with 2 liters of the dialysis buffer. The PDGF was then eluted with 0.5 M NaCl, 10 mM sodium phosphate, pH 7.4. 10 ml fractions were collected in polypropylene tubes, and assayed on a Zeiss PM6 spectrophotometer for absorbance at 280 nm. Protein containing fractions were pooled and applied 20 ml at a time to a small column of Blue Sepharose (Sigma) with approximately a 10 ml bed volume. The flow rate was controlled by a peristaltic pump and kept to 15 ml/hr. The column was washed with 100 ml of 1 M NaCl, 10 mM sodium phosphate, pH 7.4. The PDGF was eluted with 30 ml of 50% (v/v) ethylene glycol in 1 M NaCl, 10 mM sodium phosphate, pH 7.4, and collected in a polypropylene tube. This material was concentrated by lyophilization in a glass lyophilizing jar that had been previously rinsed with HCl to inactivate any residual proteins. After lyophilization the PDGF was not visible, but was able to collected by rinsing the jar carefully, three times, with 1 M acetic acid.

This reconstituted material was applied to a 2.5 X 80 cm Bio-Gel P-150 molecular sieve filtration column equilibrated with 1 M acetic acid. Elution was conducted at room temperature, at a flow rate of 10 ml / hour. Two ml. fractions were collected and assayed for protein (Lowry et al., 1951). The elution profile of this PDGF preparation was practically identical to the published elution profile of Heldin et al.

(Heldin et al., 1981a). The putative active fractions were pooled, the void volume fractions were pooled and the low molecular weight fractions were pooled. These three sets of pooled fractions were tested for PDGF activity in a modified PDGF assay, described as follows.

Within 48 hours of the addition of 10 ng/ml purified PDGF (gift of C. H. Heldin) to Swiss 3T3 cells, there is a detectable increase in cell number. 35 mm plates of Swiss 3T3 cells were plated at 10^5 cells per 35 mm. dish in serum free medium MCDB 402 with EGF, insulin, and transferrin were used to monitor PDGF activity during this final purification step. 100 μl from each of the 3 sets of pooled fractions, along with 100 μl 1 M NaOH to neutralize the acetic acid present in these fractions, was added to the 35 mm dishes of 3T3 cells. Only the suspected active fractions demonstrated any activity in this assay.

The active fraction pool was lyophilized and reconstituted with 3 ml 0.1 M acetic acid as previously described. This procedure was performed aseptically in a Baker hood to avoid filter sterilization, since PDGF is notorious for sticking to glass, plastic, etc. (Heldin et al., 1981a). The total amount of recovered protein was about 180 μg. To stabilize the PDGF, 300 μl of sterile 2% BSA was added to the 2700 μl of PDGF recovered from the lyophilization. The BSA was fatty acid free / crystalline from Sigma. 60 ng/ml of this PDGF was slightly more active than 5 ng/ml of purified PDGF from C. H. Heldin (see Results).

Reconstitution of purchased. growth factors

EGF and insulin are purchased in sterile lyophilized vials from Collaborative Research. The 100 μg of EGF is resuspended in PBS containing 0.2% BSA as follows. 5 ml of the PBS solution is aseptically drawn up into a 5 ml syringe with a 21-gauge needle. The top of the plastic vial is swabbed with 70% ethanol and pierced with the needle. The PBS solution is pushed into the vial slowly, allowing air to escape back into

the syringe every 2 ml or so. The needle is removed, and the vial is swirled and inverted. After 1 minute of gentle swirling, the top of the vial is aseptically removed with a Kim wipe dampened with 70% ethanol, and the syringe is then used to draw up the solution. The solution is distributed into small sterile polypropylene tubes in 1 ml aliquots, and these are then stored at -20^{o} C. 20 mg of lyophilized insulin is reconstituted similarly but with 4 ml of the PBS / 0.2% BSA solution, thereby yielding 5 mg/ml stock solutions. Transferrin (Human - Sigma) is dissolved at 5 mg/ml in PBS, sterilized with a Swinnex filter, and aliquoted in 1 ml aliquots into small sterile polypropylene tubes.

Fibronectin coating of dishes

35 mm dishes are covered with 1 ml 0.1% poly-D-lysine; 60 mm dishes are covered with 2 ml 0.1% poly-D-lysine. After 5 minutes at room temperature, the solution is removed and the plates rinsed with the same volume PBS. An identical volume of DME is then added, and fibronectin (90 μg for 35 mm plates, 220 μg for 60 mm plates) is added with sterile pipet tips (the idea is to use 1 μg/cm^2 fibronectin). The dishes are incubated for at least 30 minutes in a moist 37^{o} C incubator with 10% CO_2. Next, the solution is removed, and the dishes are washed once with 2 ml DME (35 mm dishes) or 4 ml DME (60 mm dishes). This DME is prepared from glass-distilled water. At this point, MCDB 402 is added (2 ml for 35 mm dishes, 4 ml for 60 mm dishes).

Addition of growth factors

In those cases where the effect of only one growth factor is being examined, gratuitous growth factors are added to the medium before it is pipetted onto dishes. The growth factor being studied is added 24 hrs later. For plating efficiency experiments, growth factors are added to the culture dishes within 1 hour of plating out the cells. Cells will sit down and spread very quickly (within 10 minutes) on fibronectin coated

dishes; allowing cells to incubate without growth factors for long periods (e.g. 12 hours) can lead to a significant loss of viability. Experiments are fed twice weekly, generally on a 4:3 schedule.

Determining the growth rates

Significant residual effects of serum are noticeable for the first day or so of these experiments. Therefore, it is advisable to count the number of cells at least 24 hours post plating. In general, the growth factor whose response is being measured is not added until this time. For plating efficiency experiments, this protocol need not be followed due to the longer incubation period and the small effect of one day's growth on a 7-day or longer experiment.

Cell numbers are determined by standard methods with a Coulter counter. In general, 2 ml of trypsin (0.025% in 0.5 mM EDTA/PBS) is added to the plates for 5 minutes. The cells will round up, but they will not detach. This unusual effect is due to the poly-D-lysine coating on the dish, and this makes it necessary for the plates to be scraped. The 2 ml suspension is then removed with a 2 ml pipet and placed in a 15 ml conical centrifuge tube. The plate is rinsed once with a small volume of isoton taken from the counting vial, and this rinse solution is added to the conical tube. Clumps are dispersed by pipeting the suspension with a 2 ml pipet gently but forcefully against the bottom of the centrifuge tube. The cells are then counted with the Coulter counter. In my original experiments, some serum was added to the dishes to neutralize the trypsin. However, this procedure made it more difficult to break up clumps of cells, and I have found that the cells remained viable without serum, for up to 1 hour.

Results

The starting point for the examination of the growth factor requirements of normal Swiss 3T3 and how they changed following SV40 transformation was the

growth factor formula developed in Gordon Sato's laboratory for the serum free growth of the Swiss 3T3 subclone 3T3-L1. 3T3-Ll will differentiate into adipocytes under certain culture conditions (Serrero et al., 1979). The growth factor formula for 3T3-Ll includes insulin, fibroblast growth factor, transferrin, and a crude rat submaxillary extract termed "gimmel" factor.

Initial attempts to grow 3T3 cells in this medium were partially successful. The parent cell line Swiss 3T3 is more growth controlled than 3T3-L1. For example, it has a 2-fold lower saturation density. In retrospect, some of the difficulty in reproducing the results of Sato's laboratory was due to the use of this more fastidious cell line. The conditions described by Sato required the initial plating of cells in 10% FCS/DME. 12-24 hours later, the plates were washed twice with serum free medium, and then the growth factors plus a 3:1 mixture of DME: F12 were added (Serrero et al., 1979). In the experiment shown in Figure 1-2, 3T3 cells were plated at various densities in 10% FCS/DME. 24 hours later, the plates were washed twice with serum free DME, and refed with either DME or 10% FCS or a 3:1 mixture of DME:F12 with added growth factors. The serum free growth of the cells plated at the lowest density was not very good, especially after a few days. However, at the higher density, the cells grew adequately for several days (Figure 1-2).

In the experiment shown in Figure 1-3, 3T3-L1 was plated at 10^4 / 35 mm dish in 10% FCS/DME. The medium was switched 12 hours later, and the growth with and without insulin was followed for 13 days. It should be noted that Sato's group plated 3T3-L1 at $2x10^4$ / 35 mm dish, and obtained exponential growth without any lag period. In the experiment shown in Figure 1-3, after a lag period, we observed exponential growth. Examination of the culture dishes during this experiment indicated that considerable loss of viability occurred during the first few days and that the exponential growth achieved was clonal.

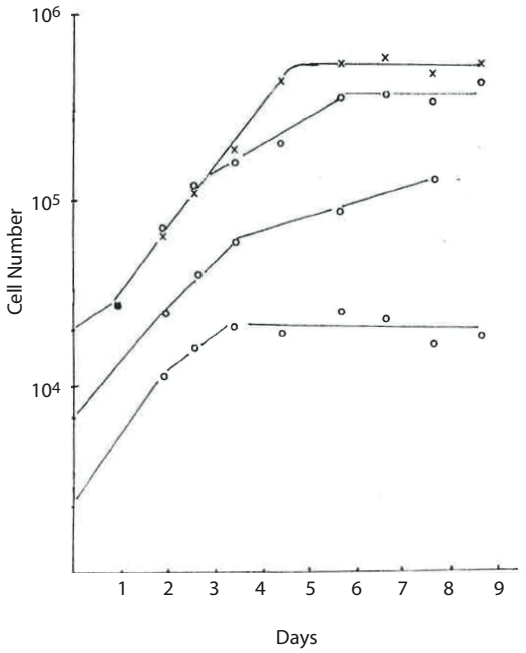

Figure 1-2. 3T3 cells were plated at various densities in 10% FCS/DME. At day 1, the cells were washed twice with serum free DME. A 3:1 mixture of DME:F12 was added with 0.5 μg insulin, 2.5 μg/ml gimmel factor, 5 μg/ml transferrin, and 2 ng/ml FGF. Circles represent serum-free medium, X's represent 10% CS.

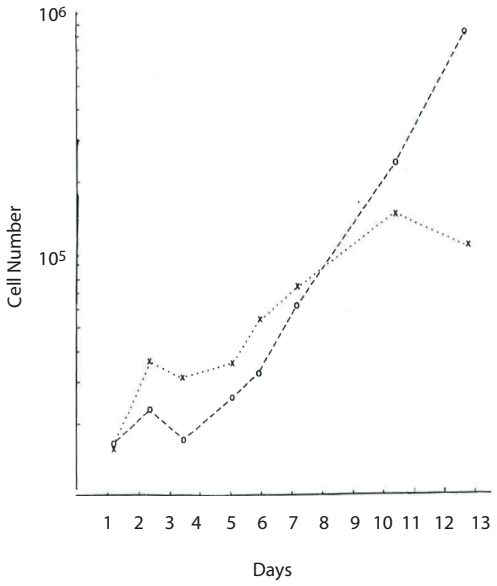

Figure 1-3. 3T3-L1 cells were plated at in 10% FCS/DME. After 12 hours, the cells were washed twice with serum free DME. A 3:1 mixture of DME:F12 was added with 0.5 μg insulin, 2.5 μg/ml gimmel factor, 5 μg/ml transferrin, and 2 ng/ml FGF. Circles represent serum-free medium with insulin, X's without insulin.

Rather than select for variants that might grow adequately at low inoculation densities, it was decided that an enriched basal medium might allow 3T3-L1 to grow exponentially at lower inoculation densities. The work of Richard Ham stressed the importance of a balanced nutrient medium in obtaining good growth at low densities. A medium had been developed specifically for mouse fibroblasts (Shipley and Ham, 1981). This medium worked well for the serum free growth of 3T3-Ll plated under the same conditions as previously described (Figure 1-4). Since our incubators are at 10% CO2, not the 5% CO2 used in Ham's laboratory, it wasn't clear if the increased concentration of sodium bicarbonate would adversely effect growth. By balancing the osmotic pressure with a

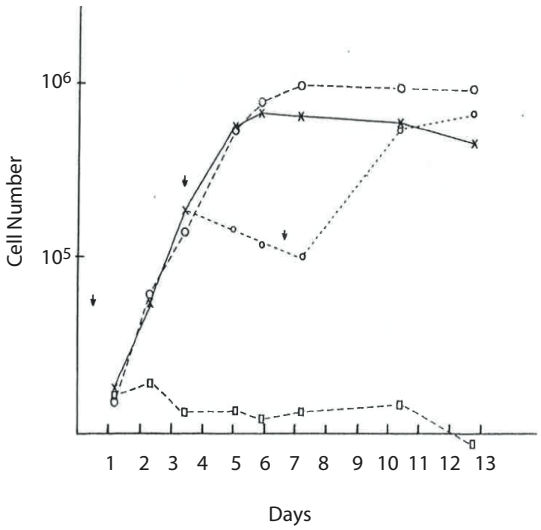

Figure 1-4. 3T3-L1 cells were plated initially in medium with 10% FCS/DME. At the time indicated by the arrow, the cells were washed twice with serum free DME, and fed with MCDB 402 containing with 1 μg insulin, 3 μg/ml gimmel factor, 5 μg/ml transferrin, and 2 ng/ml FGF (circles). The boxes represent plates receiving transferrin only. The small circles represent a growth factor shift experiment; the shift, from having growth factors to not, back to having growth factors, is indicated by the arrows.

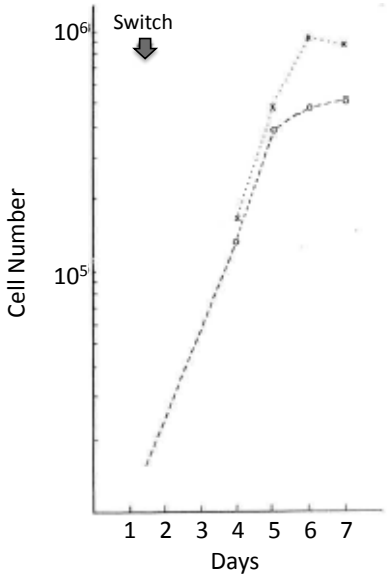

Figure 1-5. The X's represent MCDB 402 with 4.6 g/L NaCl, and the 0's represent MCDB 402 with 6.8 g/L NaCl. The conditions were otherwise the same as described in the previous figure legend..

reduction in NaCl, better growth was achieved (Figure 1-5). The serum pre incubation was not necessary if the plates were first coated for 24 hours with 10% FCS/DME, then washed twice with DME. The harvesting procedure was modified to minimize damage to the cells. The cold trypsinization method of Ham (Shipley and Ham, 1981) worked well (Figure 1-6).

However, an easier method was to wash the cells once with 0.5 mM EDTA/PBS, and then detach the cells with PBS. It was disappointing to discover that the insulin growth requirement was not that stringent in 3T3-L1 (Figure 1-7). However, the relative growth achieved was comparable to that reported by Sato (Serrero et al., 1979).

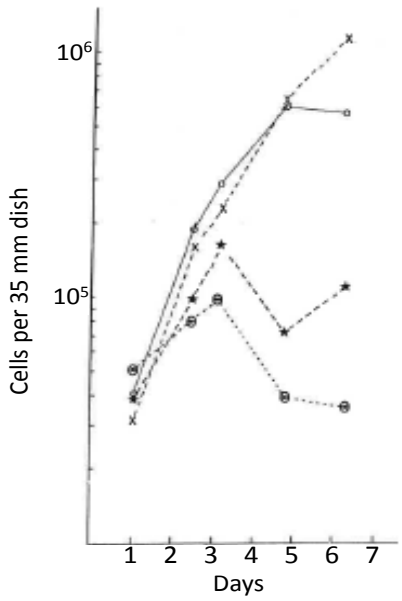

Figure 1-6. 3T3-L1 serum free growth under the same conditions described in the legend to Figure 1-4, except that the cells were plated directly into serum free medium. The cold trypsinization method of Ham was employed. X's represent cells grown in serum free medium with the full supplement, on serum coated dishes. The stars represent the growth of cells where the serum coat was replaced with 0.1% poly-D lysine. The bottom curve was obtained with fibronectin coats (2 μg/ml).

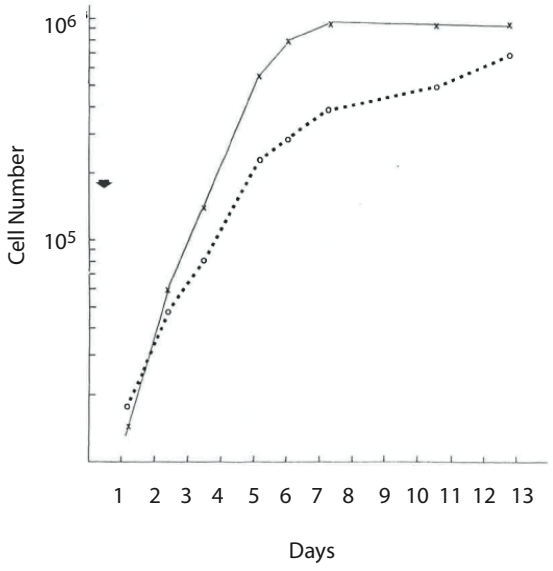

Figure 1-7. 3T3-L1 cells were plated in 10% FCS/DME. At 12 hours, the cells were washed twice with serum free DME, and fed with MCDB 402 with or without 1 μg insulin, 3 μg/ml gimmel factor, 5 μg/ml transferrin, and 2 ng/ml FGF. The X's represent cells grown with insulin, the O's without insulin.

A much more dramatic insulin requirement was observed when 500 cells were plated and visible colonies scored 10 days later (Table 1-1). As will be discussed in depth in a later chapter, this density dependence of the stringency of the insulin requirement is likely to be due to the fact that fibroblasts secrete insulin-like growth factors (Atkison and Bala, 1981; Clemmons et al., 1981).

If growth was carried out for 14 days, the difference was less marked (Table 1-2). Insulin, gimmel factor, and FGF all had an effect on colony formation. Of particular interest in this experiment was that 0.1% FCS, and 0.1% CS to a lesser extent, could replace insulin for optimal colony formation. An initial scan of various cell lines gave some indication that a

Table 1-1. Colony Formation of 3T3-L1 in Serum Free Medium I

	Colonies / 500 Cells Plated	
Condition	**Plate 1**	**Plate 2**
10$ FCS/DME	130	117
Full Mix	119	155
Full Mix + Vasopressin	129	149
2X of the above	133	111
Full Mix Minus Insulin	3	4
Full Mix / DME:F12	0	0

3T3-L1 cells were grown for 10 days as described in the text. Visible colonies were scored following fixation with 10% formalin/PBS and staining with Giemsa stain. MCDB 402 was supplemented with 1 μg/ml insulin, 5 μg/ml transferrin, 2 ng/ml FGF, and 3 μg/ml gimmel factor.

Table 1-2. Colony Formation of 3T3-L1 in Serum Free Medium II

Condition	Colonies / 100 Cells Plated
10% Calf Serum	31
Full Mix	59
Full Mix - FGF	33
Full Mix - Gimmel Factor	10
Full Mix - Insulin	12
Transferrin only	0
Full Mix - Ins + 0.1% FCS	67
Full Mix - Ins + 0.1% CS	38

3T3-L1 cells were grown for 14 days as described in the text. Visible colonies were scored following fixation with 10% formalin/PBS and staining with Giemsa stain. MCDB 402 was supplemented with 1 μg/ml insulin, 5 μg/ml transferrin, 2 ng/ml FGF, and 3 μg/ml gimmel factor.

reduced insulin requirement might be associated with neoplastic transformation . As shown in Table 1-3, the parental cell line 3T3 had a more stringent insulin requirement than 3T3-L1. 3T3-L1 was similar to the 'minimal' transformants SVR95 and SVR57. These 3T3 derivatives were isolated after SV40 infection, but were T-antigen negative (Risser and Pollack, 1974). Nevertheless, they displayed a reduced serum requirement and grew to somewhat higher saturation densities than 3T3 (Risser and Pollack, 1974). A much greater difference was observed in fully transformed derivatives of 3T3 like SV101 and Polyoma-3T3 (Table 1-3).

These cell lines had a 30-fold higher relative plating efficiency than 3T3. 3T6, a cell line established from mouse embryo fibroblasts passaged at high densities (Todaro and Green, 1966a), also had a 30-fold higher relative plating efficiency.

Since 3T6 does not grow in soft agarose or methocel, this dissociates the reduced insulin requirement from anchorage independence. The SV40 dl884 transformed derivative of 3T3, which lacks 17K t -antigen, had only a 15-fold increased relative plating efficiency, similar to the 'intermediate' transformants SVR13 and SVR18. 'Intermediate' transformants do not contain as much T-antigen as full transformants (Risser and Pollack, 1974).

Table 1 3. Insulin Requirement of Various Mouse Fibroblasts I

Cell Line	Plating Density	Days Cultured	10% CS	Full Mix	Full Mix - Insulin	R.P.E.
Swiss 3T3	100	15	82 ± 15	25 ± 3	0	< 0.01
Swiss 3T3	500	11	27 ± 3	8 ± 1	0.1 ± 0.3	0.01
3T3-L1	100	14	40 ± 8	36 ± 11	3 ± 2	0.08
SV101	100	12	60 ± 7	58 ± 4	20 ± 7	0.34
Py-3T3	100	14	9 ± 5	10 ± 3	3 ± 2	0.3
3T6	100	12	38 ± 12	29 ± 8	10 ± 5	0.34
884D	100	15	73 ± 6	26 ± 3	4 ± 6	0.15
SVR18	100	15	71 ± 3	40 ± 5	6 ± 0	0.15
SVR13	100	14	65 ± 19	12 ± 1	2 ± 0	0.17
SVF65	100	15	39 ± 3	22 ± 1	2 ± 1	0.09
SVR57	500	12	confluent	14 ± 1	1 ± 1	0.07
LSSVl	100	12	36 ± 1	31 ± 3	2 ± 2	0.06
AγSV4	100	17	confluent	22 ± 1	0	< 0.01
AγSV4	500	10	29 ± 5	13 ± 3	0.1 ± 0.3	0.01

Various cell lines were tested for their insulin requirement in MCDB 402 with the supplements listed in the legend to Table 1-1.

Of particular interest was the reacquisition of the insulin requirement in the serum-sensitive revertants of SV101, LSSV1, and AγSV4. The insulin requirement of AγSV4 was very low, comparable to the insulin requirement of Swiss 3T3 (Table 1-3).

These preliminary results were confirmed and extended in a subsequent experiment. 10^3 cells were plated with or without insulin and visible colonies were scored 7 days later. Normal Balb/3T3 and Swiss 3T3 displayed strong insulin requirements (Table 1-4).

The reduced insulin requirement of the transformed cell lines 3T6 and SV101 observed previously were also apparent in this experiment. Additionally, Sarcoma 180, a tumor derived cell lire, and KA31T, a Kirsten Murine Sarcoma virus transformed derivative of Balb/3T3, displayed reduced insulin requirements (Table 1-4), indicating that a reduced insulin requirement may be a general feature of transformed fibroblasts.

Table 1-4. Insulin Requirement of Various Mouse Fibroblasts II

Cell Line	10% CS	Full Mix	Full Mix - Insulin	R.P.E.
Swiss 3T3	13 ± 1	8 ± 1	0.4 ± 0.1	0.05
Balb 3T3	10 ± 1	9 ± 5	0.2 ± 0.1	0.02
AySV4	14 ± 3	10 ± 1	0.3 ± 0.2	0.03
SVR95	28 ± 1	15 ± 2	0.4 ± 0.1	0.03
SVR57	15 ± 1	8 ± 2	0.9 ± 0.3	0.11
SVR13	nd	14 ± 1	1.4 ± 0.4	0.10
884D	32 ± 1	26 ± 3	5 ± 1	0.19
SV101 (B8)	nd	14 ± 1	2.5 ± 0.3	0.18
MR	15 ± 4	11 ± 2	5 ± 1	0.45
Sarcoma 180	24 ± 1	24 ± 3	9 ± 1	0.38
3T6	32 ± 1	24 ± 1	8 ± 1	0.33
KA31T	15 ± 1	16 ± 8	8 ± 1	0.50

Various cell lines were plated onto serum coated 60 mm dishes (1000 cells / dish) containing 4 ml MCDB 402 with or without 10 μg/ml insulin, along with 10 ng/ml FGF, 5 μg/ml gimmel factor, and 5 μg/ml transferrin.1 week later, the number of visible colonies was determined.

A subclone of SV101 that had been treated with BUdR (B8) had a stronger insulin requirement than SV101. Again, the dl884 transformant and the intermediate SV40 transformant had modestly reduced insulin requirements (Table 1-4). The distinction previously observed between the minimal transformants and 3T3 was not reproducible since the cell line SVR95 had a low relative plating efficiency similar to 3T3 (Table 1-4). The SV101 serum-revenant AγSV4 again displayed a stringent insulin requirement. AγSV4 formed colonies at 1% efficiency in methocel, indicating that the anchorage requirement and the insulin requirement are dissociable.

We wanted to determine whether the strong insulin requirement for colony formation was also reflected in the relative growth rate of cells plated at higher densities. 10^4 cells were plated onto 60 mm dishes and the number of doublings with and without insulin was determined four days later. The relative growth rate was roughly two-fold higher for the transformed cell lines 3T6, SV101, and Sarcoma 180

than it was for Swiss 3T3 (Table 1-5). AγSV4 had a stronger insulin requirement, similar to 3T3.

Table 1-5. Insulin Requirement of Various Mouse Fibroblasts III

	Relative Growth Rate		
Cell Line	**Full Mix**	**Ins**	**(Ins/±Ins)**
Swiss 3T3	2.8 ± 0.2	1.0 ± 0.1	0.36
AγSV4	4.1 ± 0.1	1.6 ± 0.1	0.39
3T6	4.2 ± 0.3	2.8 ± 0.2	0.67
884D	3.7 ± 0.1	2.1 ± 0.2	0.57
Sarcoma 180	4.4 ± 0.1	3.6 ± 0.1	0.82
SV101	4.1 ± 0.3	3.3 ± 0.1	0.80

These cell lines were plated as described in the text onto 60 mm serum coated dishes, containing medium with or without insulin. Conditions were identical to those described in the legend to Table 1 4.

While these findings confirmed our suspicions that transformed cells displayed a reduced insulin requirement, we were somewhat concerned about the effect of the serum coat. Growth factors have been shown to be mitogenically active when non-specifically adhered to culture dishes (Smith et al., 1982). We also wanted to replace the crude "gimmel" factor. It was possible that the insulin requirement of transformed cells would be the same as the insulin requirement of normal cells in the absence of any insulin or insulin-like molecules.

An important step in using fibronectin was to pre-coat the dishes with poly-D-lysine. The importance of following this sequence was discovered by Sato for the growth of C6 glioma cells (Wolfe et al., 1980). As seen in Table 1-6, fibronectin coating supported the growth of Swiss 3T3 cells in a 3-day assay with 62% efficiency relative to serum coating. Although fibronectin coating was not much better than poly-D-lysine alone (Table 1-6), cells incubated for 7 days in the absence of fibronectin demonstrated some loss of cell viability, whereas with fibronectin they did not.

Substituting thrombin, EGF, and prostaglandin F2-alpha (PGF2α) for gimmel factor worked well (Table 1-6). Thrombin had little effect, and PGF2 α worked well for

four days but by 7 days it caused the cells to pile up into organized striations, and eventually it appeared as if some of the cells differentiated into chondrocytes. With 5-azacytidine, 3T3 cells can be induced to differentiate into chondrocytes. Whether these cells did differentiate or not, this phenomenon warranted the discontinuation of the use of PGF2α.

Table 1-6. Revision of Serum Free Medium Mix

Condition	Doublings	Relative Growth Rate
Full Mix	2.4	
Full Mix - Ins	0.8	.33 (Ins/+Ins)
New Mix	2.6	
New Mix - Thrombin	2.5	.96 (Thr/+Thr)
New Mix - PGF2	2.3	.88 (PGF2 /+PGF2)
New Mix - Ins	0.9	.35 (Ins/+Ins)
FN Coat	1.6	.62 (FN Coat/Serum)
No FN	1.3	.81 (FN/+FN)

3T3 cells were plated under the conditions described in the text.

The final step in the development of the serum free medium was the addition of PDGF in place of FGF. PDGF and FGF are interchangeable in many short term assays (Scher et al., 1978). However, 10 ng/ml PDGF was more potent than 10 ng/ml FGF for the growth of Swiss 3T3 cells in a 3-day growth assay (Table 1-7). 60 ng/ml of the PDGF I partially purified was as active as 20 ng/ml purified PDGF. This is in agreement with the relative potencies of purified and partially purified PDGF as reported by Heldin (Heldin et al., 1981a).

Table 1-7. Purified PDGF is Superior to FGF but Reduces the Insulin Requirement

Condition	Doublings	Relative Growth Rate
Insulin, EGF, FGF	1.5	
EGF, FGF	0.5	0.33
Insulin, EGF, plus 10 ng/ml PDGF	2.6	
The above minus insulin	2.1	0.81
Insulin, EGF, plus 20 ng/ml PDGF	3.2	
Insulin, EGF, plus 60 ng/ml PDGF	3.3	
The above minus insulin	2.6	0.79

3T3 cells were plated onto poly-D-lysine/fibronectin coated 60 mm dishes containing 4 ml MCDB 402 supplemented with or without 10 μg/ml insulin, 10 ng/ml FGF, 40 ng/ml EGF, and various amounts of PDGF.

Unfortunately, PDGF reduces the insulin requirement (Table 1-7). This is most likely a result of the ability of PDGF to greatly stimulate the secretion of somatomedin C (IGF-I) in fibroblasts (Clemmons and Van Wyk, 1981). Thus, more sensitive assay of the insulin requirement necessitated the use of FGF instead of PDGF.

Swiss 3T3 cells grow with a doubling time of approximately 22 hours with PDGF, EGF, insulin, and transferrin (Figure 1-8). This growth rate is comparable to the growth rate obtained with 10% FCS. This defined medium supported clonal growth as well and was strictly dependent upon PDGF, strongly dependent upon insulin, and not appreciably affected by EGF (Figure 1-9).

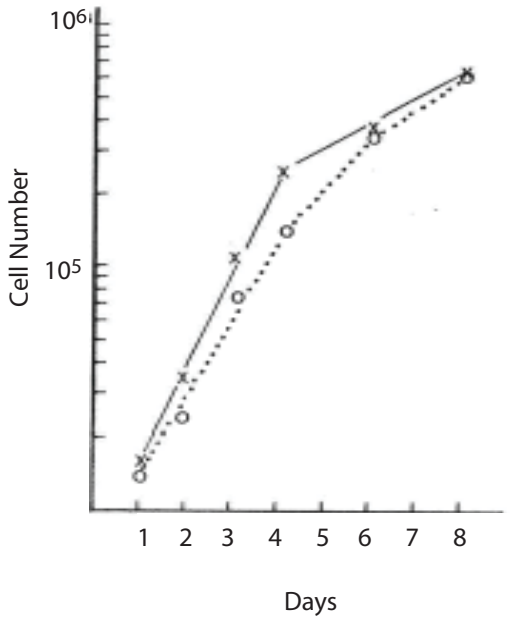

Figure 1-8. Swiss 3T3 cells were plated directly into defined medium supplemented with 60 ng/ml partially purified PDGF, 40 ng/ml EGF, 10 μg/ml insulin, and 5 μg/ml transferrin. The 35 mm dishes were treated with poly D lysine and fibronectin. 0's represent cells grown in defined medium, X's represent cells grown in 10% FCS/DME.

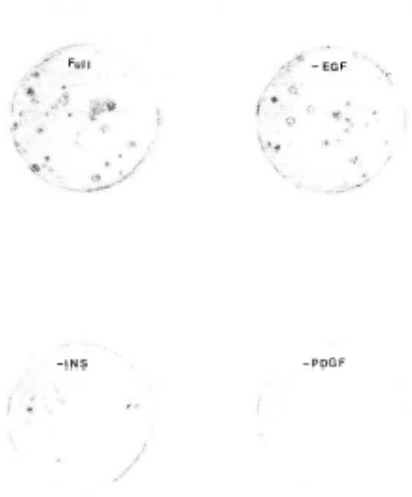

Figure 1-9. Swiss 3T3 cells were plated at 200 cells/ 60 mm dish under the defined conditions described in the legend to Figure 1-8. The effect of the various growth factors on clonal growth was examined. PDGF was clearly the most stringently required growth factor for clonal growth. EGF had a barely discernible effect. Insulin is not absolutely required for clonal growth in the presence of PDGF, but it clearly has an effect. The cells were grown for 13 days before fixation. Standard colonies growing in 10% FCS would be three to four

DISCUSSION

Starting with the partially defined medium of Sato 's group, a totally defined medium for the growth of Swiss 3T3 cells was developed. The important steps in this development were (1) the replacement of the DME:F12 basal medium with MCDB 402, (2) the deletion of the serum coating and its replacement with sequential poly-D-lysine/fibronectin coating, (3) the deletion of gimmel factor, and (4) the substitution of PDGF for FGF. Throughout the development of these conditions, a difference was noted between transformed fibroblasts and normal fibroblasts in terms of their insulin growth requirement. As discussed in a later chapter, this difference holds true with PDGF, but it is lessened due to the ability of PDGF to induce IGF secretion.

CHAPTER 2

ALTERED GROWTH REQUIREMENT FOR IGF-1 IN TRANSFORMED FIBROBLASTS

Abstract

In a totally defined serum free medium, which utilizes insulin, EGF, and FGF, normal 3T3, cells grew very slowly in the absence of insulin. SV40 transformed 3T3 cells and other transformants grew significantly faster in the absence of insulin. This reduced growth requirement for insulin was shown to reflect a reduced requirement for insulin like growth factor I (IGF-I). Dose response studies with insulin, IGF-I, and MSA (an IGF-II analogue) indicated that the growth promoting effect of insulin in this system was in all probability mediated by IGF-I receptors. The reduced requirement for IGF-I that we have found in transformed fibroblasts may allow these cells *in vivo* to escape from the pituitary growth hormone/IGF-I regulatory system. Additionally, dose response studies revealed that SV40-transformed 3T3 cells were 10 fold more sensitive to both insulin and IGF-I than normal 3T3 cells. This may reflect an alteration in the affinity of IGF-I receptors upon transformation. Alternatively, it may reflect an alteration in a secondary mediator of IGF-I induced mitogenesis.

Introduction

The control of fibroblast growth in vivo is known to be affected by two growth factors, platelet derived growth factor (PDGF) and the somatomedin IGF-I. In the wounding process, platelets release PDGF, which stimulates fibroblasts to migrate into the wound and begin proliferation (Ross and Vogel, 1978). Additionally, PDGF is a potent inducer of somatomedin C (IGF-I) production by cultured fibroblasts (Clemmons

et al., 1981). The production of IGF-I probably augments the mitogenic response to PDGF (Clemmons and Van Wyk, 1981). *In vivo*, excessive secretion of pituitary growth hormone elevates plasma levels of IGF-I to abnormally high levels (Daughaday et al., 1975). In children, such a condition will cause gigantism (Van Wyk and Underwood, 1975). After bone ossification is complete, the pathological condition of acromegaly results, in which both fibroblast and osteoblast proliferation are uncontrolled (Van Wyk and Underwood, 1975).

Growth of fibroblasts in vitro can be controlled by varying the parameters of substrate attachment (Folkman and Moscona, 1978), cell density (Todaro and Green, 1963), or the serum concentration (Holley and Kiernan, 1968). Replacing serum with hormones allows for the study of response to individual growth factors in a controlled environment (Barnes and Sato, 1980). Previously, others have noted a reduced epidermal growth factor requirement in transformed hamster fibroblasts (Cherington et al., 1979). In this report, we examine the growth response of normal and transformed cells to insulin and insulin like growth factors.

Materials and Methods

The methods employed are described fully in Chapter 1, and the experimental conditions are described in the legends.

Results

Insulin stimulated the growth of both SV40 transformed 3T3 (clone SV101) and normal 3T3 in a 4-day growth assay. As seen in Figure 2-1, the inclusion of insulin resulted in a 2-3 fold higher final cell number for both cell lines. However, when the rates of growth were analyzed, it was apparent that insulin was more stringently required by 3T3 than it was by SV101 (Figure 2-2). Thus, the absence of insulin did not

dampen the growth rate of SV101 to the same extent that it decreased the growth rate of 3T3.

3T3 required supraphysiological concentrations of insulin for maximal growth (Figure 2-3). The half maximal dose was approximately 20 nM, which is well out of the range expected if insulin was stimulating growth via the high affinity (K_D = 0.1 nM) insulin receptor (Czech,

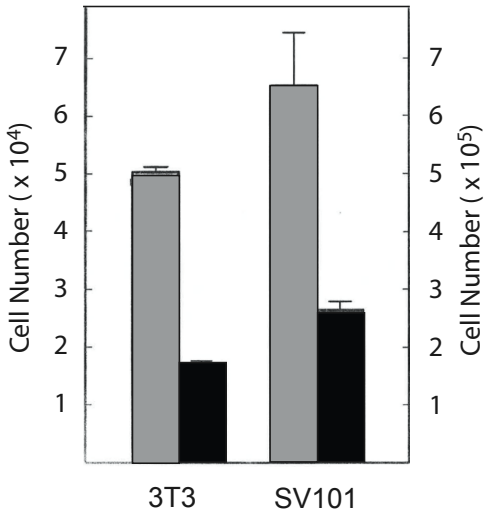

Figure 2-1. Swiss 3T3 and SV101 were plated onto fibronectin coated dishes at a density of 2 x 10^4 cells / 35 mm dish using 2 ml of MCDB 402 supplemented with 5 ug/ml transferrin, 10 ng/ml FGF, and 40 ng/ml EGF. The grey bars indicate cells receiving 20 μg/ml insulin, black bars without. After 4 days, cells were counted. The scale for 3T3 is shown on the left, SV101 on the

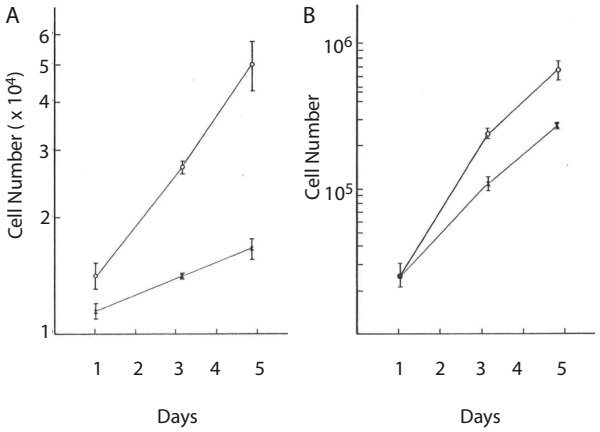

Figure 2-2. Swiss 3T3 (Panel A) and SV101 (Panel B) were plated onto fibronectin coated dishes at a density of 2 x 10^4 cells / 35 mm dish using 2 ml of MCDB 402 supplemented with 5 μg /ml transferrin, 10 ng/ml FGF, and 40 ng/ml EGF. Circles represent growth with 20 μg/ml insulin, X's represent growth without.

1982). SV101 was largely independent of insulin in terms of its relative growth rate (Figure 2-3). Even so, its half maximal dose for its insulin growth response was approximately 10 fold lower than the ED_{50} for 3T3. This altered sensitivity of transformed fibroblasts to insulin has been observed in other systems (Rockwell et al.,

1980; Taub et al., 1981; Temin, 1967). However, with the exception of Temin, these reports did not examine whether transformed cells grew relatively better than normal cells in the complete absence of insulin.

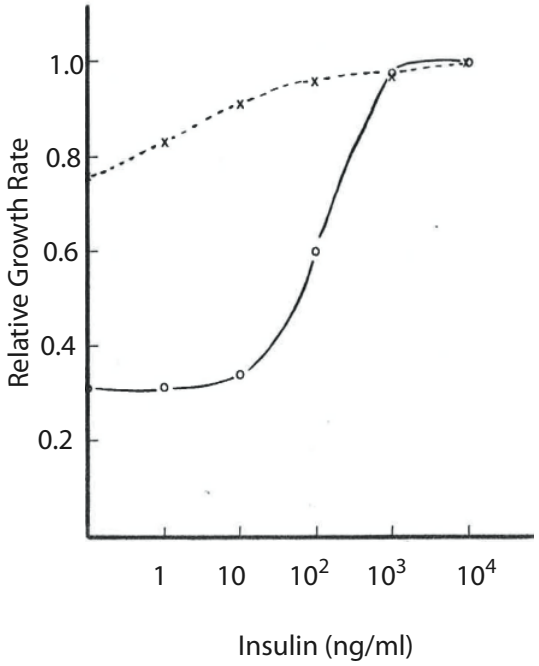

Figure 2-3. Dose-response studies with insulin. Relative doublings of cells with and without insulin are reported for SV101 (X's) and 3T3 (Circles). Cells were grown for four days under conditions as described in the legend to Figure 2-1.

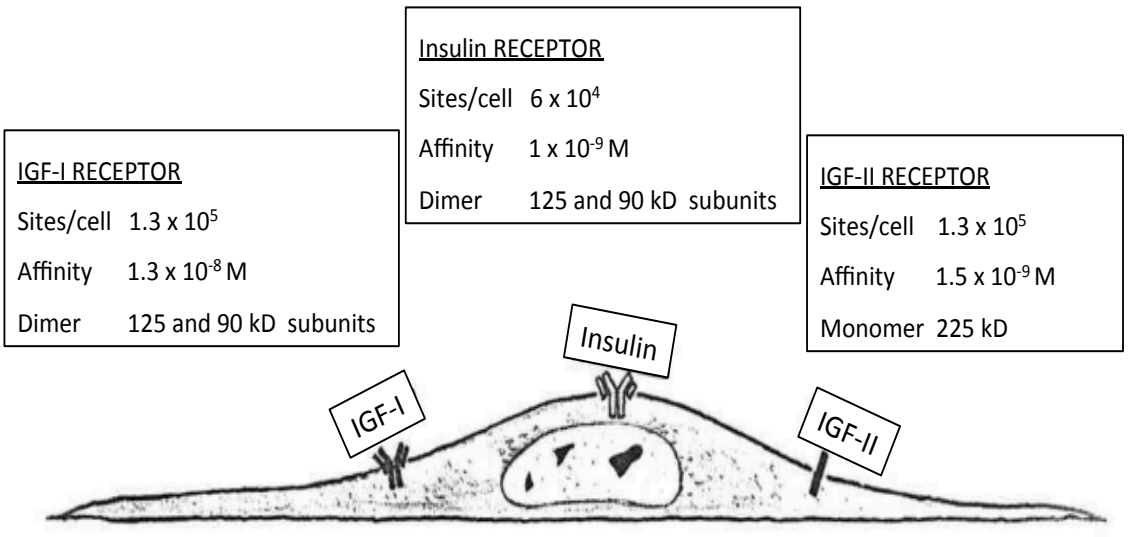

Figure 2-4. The insulin-hormone family of receptors on 3T3 cells adapted from Czech et al., 1982.

The high ED_{50} of 3T3 indicated that the growth response to insulin might be mediated by other receptors. Czech has shown that Swiss 3T3 cells express three distinct receptors that bind to peptides belonging to the insulin family of hormones (Czech, 1982). These receptors are the high affinity insulin receptor, the IGF-I receptor, and the IGF-II receptor (Figure 2-4). The insulin and IGF-I receptors have structures similar to IgG molecules, whereas the IGF-II receptor is a single polypeptide chain (Czech, 1982).

Dose response studies of 3T3 and the IGF-II analogue MSA indicated that MSA was not significantly more potent than insulin for stimulating growth (Figure 2-5).

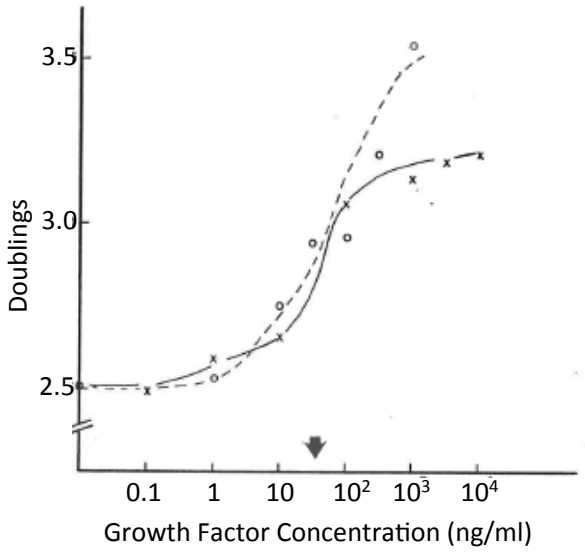

Figure 2-5. Dose response studies with insulin and MSA on 3T3 cells. The conditions were identical to the conditions described in the text and the legend to Figure 2-3 with one important exception. In place of FGF, I used 1 μg/ml of a preliminary fraction of PDGF. This was before I became aware of the fact that PDGF stimulates IGF-I production and thereby reduces the insulin requirement. Note that the half maximal effect is somewhat reduced in the presence of PDGF, although this slight reduction is less dramatic than the 10 fold reduction of SV101. Circles represent cells grown with MSA, X's represent cells grown with insulin.

This same observation has been reported for human fibroblasts (King and Kahn, 1981). However, IGF-I was approximately 100 fold more potent than insulin for stimulating the growth of 3T3 cells (Figure 2-6), in agreement with results obtained for stimulation of DNA synthesis in human fibroblasts (King and Kahn, 1981).

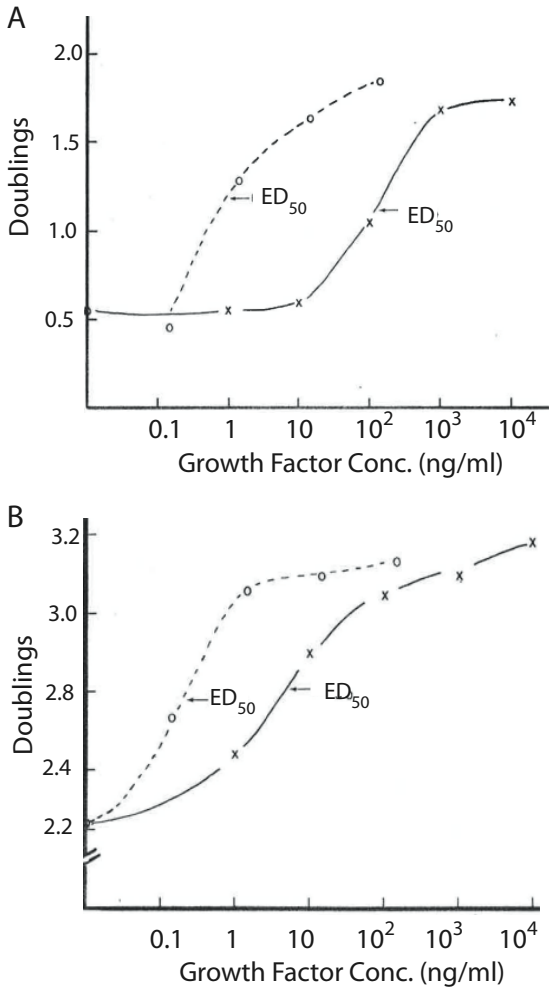

Figure 2-6. Dose-response studies with insulin and IGF-1 with Swiss 3T3 (Panel A) and SV101 (Panel B). Doublings of cells with (circles) and without insulin (X's) are reported. Cells were grown for four days under conditions as described in the legend to Figure 2-1.

Thus, it is likely that IGF-I receptors are the mediators of the growth response to insulin and insulin-like molecules in 3T3 fibroblasts. _Confirmation of this hypothesis will have to await blocking studies with specific antibodies.

Similarly, IGF-I was approximately 100 fold more potent than insulin for stimulating the growth of SV101 (Figure 2-6). The ED_{50} of SV101 for IGF-I was approximately 10 fold lower than the IGF-I ED_{50} for 3T3. Apparently, SV40 transformation increases the sensitivity of cells to IGF-I. However, SV101 grew relatively well in the complete absence of IGF-I, whereas 3T3 did not.

As seen in Table 2-1, a reduced insulin requirement appears to be a general feature of transformed fibroblasts. The spontaneous transformant 3T6, the tumor derived cell line 95TUMOR (Freedman et al., 1975), and the Kirsten Murine Sarcoma virus transformed NIH DT all displayed a reduced requirement for insulin (Table 2-1).

Table 2-1. Reduced Insulin Requirement in Transformed Fibroblasts

		Doublings		Relative Growth Rate
Cell Line	**Parent**	**Ins,EGF,FGF**	**EGF,FGF**	
Swiss 3T3	mouse embryo	1.8 ± 0.3	0.6 ± 0.1	.30 ± .06
NIH 3T3	mouse embryo	1.5 ± 0.1	0.5 ± 0.1	.35 ± .03
3T6	mouse embryo	4.0 ± 0.2	2.1 ± 0.2	.52 ± .06
NIH DT	NIH 3T3	5.4 ± 0.1	5.5 ± 0.3	.98 ± .02
M01	Swiss 3T3	4.7 ± 0.1	3.4 t 0.1	.73 ± .05
Swiss 3T3*	mouse embryo	1.7 ± 0.2	0.3 ± 0.2	.18 ± .06
95TUMOR**	Swiss 373	2.8 t 0.2	1.2 ± 0.1	.43 ± .02

Cells were plated onto fibronectin coated dishes (20,000 cells/ 35 mm dish) with 2 ml MCDB 402 supplemented with 5 μg/ml transferrin, 10 ng/ml FGF and 40 ng/ml EGF. 24 hours later duplicate plates were counted, and 20 μg/ml insulin was added to some of the plates. 4 days later, duplicate plates for each condition were counted, and the counts were converted to doublings. The values reported are the means ± the standard errors. *10 ng/ml vasopressin was used in place of FGF, and 20 μg/ml insulin was added to some of the plates. 4 days later, duplicate plates for each condition were counted, and the counts were converted to doublings. The values reported are the means ± the standard errors. **10 ng/ml vasopressin was used in,place of FGF, and the inoculation density was 5000 cells /35 mm dish.

Temin working with Rous sarcoma virus transformed chick embryo fibroblasts originally discovered this phenomenon. Our results indicate that the reduced insulin requirement actually reflects a reduced requirement for IGF-I.

Discussion

The central role of the growth hormone/IGF-I system in the regulation of growth after birth has been extensively documented (Van Wyk and Underwood, 1980). IGF-I levels are tightly regulated by an intricate system of feedback loops involving the hypothalamus and the pituitary (Figure 2-8).

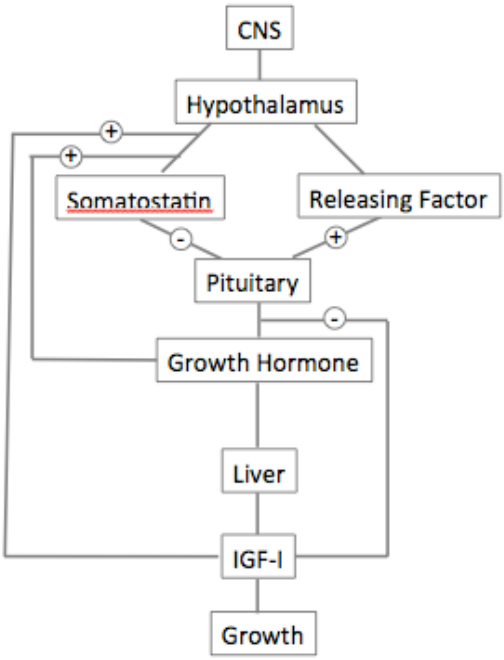

Figure 2-7. Feedback inhibition in the regulation of IGF-I levels. Although the liver is currently the suspected site of IGF-I production, this issue has never been conclusively settled. The existence of a releasing factor has been recently discovered (Brazeau et al., 1982). Pluses represent positive stimulation, minuses represent negative stimulation. This is adapted from (Berelowitz et al., 1981).

Fibroblastic cells are especially responsive to IGF-I, and elevated levels of IGF-I can cause inappropriate proliferation of fibroblasts. Our results indicate that transformed fibroblasts proliferate relatively well in the absence of IGF-I. By analogy with the uncontrolled fibroblast proliferation found in acromegaly, the uncontrolled proliferation of fibrosarcomas may be due at least in part to the loss of a strong IGF-I requirement.

Although SV101 grew relatively well in the absence of insulin or IGF-I, it was 10 fold more sensitive than 3T3 in dose response experiments for both growth factors. This increased sensitivity could be due to an alteration in the affinity of the IGF-I receptor; however, this would not explain why SV101 grows relatively well in the complete absence of IGF-I. Alternatively, there may be an alteration in a protein or proteins involved in mediating the IGF-I receptor signal. We favor this latter hypothesis for the following reasons: (1) the IGF-I ED_{50} for 3T3 was approximately 0.2 nM; this is lower than the reported K_D of the IGF-I receptor for 3T3 (11 nM). This type of shift is very common in hormone receptor induced effects, and presumably reflects the existence of a secondary mediator of hormone action (e.g. cAMP mediates the epinephrine signal) (Strickland and Loeb, 1981), (2) compared to 3T3, SV101 behaves as though it is responding to IGF-I in the complete absence of IGF-I. Additionally, its ED_{50} is approximately 10 fold lower. A single alteration in a secondary mediator could account for both of these changes. The alteration in the actin cytoskeleton we have previously observed in SV101 may underlie this phenomenon (Pollack et al., 1975), as the cytoskeleton has been clearly implicated in the mediation of growth factor induced mitogenesis (Maness and Walsh, 1982).

CHAPTER 3

ANALYSIS OF THE REDUCED INSULIN, EGF, AND PDGF REQUIREMENTS OF SV40-TRANSFIORMED 3T3 CELLS

Abstract

In order to examine the basis for the reduced serum dependence of SV40 transformed fibroblasts, we have measured the insulin, epidermal growth factor (EGF), and platelet derived growth factor (PDGF) requirements for the serum free growth of normal 3T3 and SV40⁻transformed 3T3 cells. 3T3 had a stringent requirement for insulin and PDGF but only a modest requirement for EGF. All three of these requirements were diminished or abolished in SV40 3T3. Analysis of partial and full revertants of SV40 3T3 revealed that the loss of the PDGF requirement was dissociable from a reduced serum dependence; rather, it co segregated with the loss of density dependent growth inhibition. Similarly, only one of the serum sensitive revertants regained a 3T3 like EGF requirement, although both serum sensitive revertants displayed significantly altered EGF binding. Only the diminished insulin requirement was directly linked with reduced serum dependence. We present evidence that one serum sensitive revertant has suffered a cellular mutation that specifically effects its requirement for insulin.

Introduction

Transformation by SV40 and other oncogenic agents often reduces the high serum growth requirement of normal fibroblasts (Dulbecco, 1970; Holley and Kiernan, 1968; Risser and Pollack, 1974; Smith et al., 1971; Temin, 1966). Several reports have led to the conclusion that a reduced serum requirement results from the loss of a requirement for specific serum growth factors. For example, Rous sarcoma virus transformation of chick embryo fibroblasts reduced their requirement for the insulin replaceable activity in serum (Temin, 1967), and abortive SV40 infection of normal mouse 3T3 cells stimulated DNA synthesis in the absence of

platelet derived growth factor and the insulin like growth factor somatomedin C, both of which were necessary for the stimulation of uninfected cells (Scher et al., 1978; Stiles et al., 1979). Additionally, Cherington et al. (Cherington et al., 1979) discovered that a reduced EGF requirement was associated with tumorigenicity in a set of hamster fibroblast cell lines.

Not all of these reported changes need be directly related to reduced serum dependence. Transformed cells often exhibit other alterations in growth control, such as the loss of density dependent growth inhibition and the loss of a normal anchorage requirement (Risser and Pollack, 1974). If the loss of a specific growth factor requirement is directly linked to reduced serum dependence, then the two phenotypes should co segregate upon reversion. We and others have isolated revertants of SV40 transformed cells that have regained a normal serum dependence (Toniolo and Basilico, 1975; Vogel and Pollack, 1973). Since wild type SV40 virus can be rescued from these revertants, and since they continue to express SV40 T-antigen, it is thought that these revertants harbor a cellular mutation that reverses the phenotype of a reduced serum requirement (Vogel and Pollack, 1973; Toniolo and Basilico, 1975). For some of these revertants, the putative cellular mutation effects the serum requirement without affecting the ability to grow without anchorage to a solid substrate (Vogel and Pollack, 1973).

Previously, we discovered that intracellular cAMP concentrations responded specifically to growth regulation by serum, and not to density dependent growth regulation (Oey et al., 1974). Serum-sensitive revertants regained the property of responding to serum deprivation by a sharp rise in intracellular cAMP levels. In contrast to this linkage of cAMP response to serum dependence, serum-sensitive revertants did not regain the "normal" response to serum deprivation of a reduction in the transport rates of either glucose or amino acids (Dubrow et al., 1978). This finding is in agreement with other reports that cell growth is dissociable from increased nutrient uptake (Thrash and Cunningham, 1974; Weber and Friis, 1979). In this paper, we report that the reversion to serum dependence is linked to the reacquisition of a specific growth factor requirement, but dissociable from the requirement for other growth factors.

It is well established that growth factors function by binding to specific cellular receptors (Heldin et al., 1981b), and current evidence supports the notion that the mitogenic response is mediated by specific receptors and not by internalized growth factor molecules (Schreiber et al., 1981). Transformation to growth factor independence might be accomplished by any of a number of mechanisms that mimic the activity of the exogenous growth factor, such as secretion of a homologous growth factor, direct perturbation of growth factor receptors, or constitutive expression of an intracellular event that is normally triggered by receptor activation. Revertants that have suffered cellular mutations would be a potentially powerful tool in the analysis of the mechanisms by which transformed cells escape growth factor requirements.

Materials and Methods

Cell Lines

Swiss 3T3, SV40 transformed Swiss 3T3 (clone SV101), and the revertants derived from SV101 were maintained in 10% FCS/DME (vol/vol) with 100 units/ml penicillin and 100 μg/ml streptomycin. SVIH1 was cloned from SV40 infected Swiss 3T3 (Pollack et al., 1968). FLSV101 (FLSV) was derived from SV101 by FUdR negative-selection at high density (Pollack et al., 1968). The serum revertants AγSV101-4 (Aγ4) and LSSV101-1 (LS1) were derived from SV101 by BUdR negative selection at low density in agamma globulin depleted serum and low (1%) serum, respectively (Vogel and Pollack, 1973). Kirsten murine sarcoma virus transformed Aγ4 (Ki-Aγ4) was isolated from a morphologically altered, densely growing colony following Ki-MuSV infection of Aγ4 (Vogel and Pollack, 1974). NIH-3T3 cells were obtained from M. Wigler, and NIH-DT cells, a Ki-MuSV transformed derivative of NIH 3T3 isolated in E. Scolnick's laboratory, were obtained from V. Vogt. All cell lines were found to be free of mycoplasma contamination by the fluorescent method of Chen (Chen, 1977).

The published properties of these cell lines (anchorage requirement, serum requirement, and saturation density) were confirmed recently in this laboratory. However, the use of fetal calf serum in place of calf serum proved unsatisfactory for the measurement of the serum requirement. All cell lines grew significantly faster in 1% FCS than they did in 1% CS. The ratios of doubling times in 1% calf serum to 10% calf serum were as reported, except that previously Ki-Aγ4, like Aγ4, did not grow at all in 1% CS (Vogel and Pollack 1974), and in recent experiments it retained a normal serum requirement but like 3T3 grew slowly in 1% CS. Saturation densities in 10% FCS were within a 0.4 fold range of published values (Vogel and Pollack, 1974). Plating efficiency in methocel with 10% FCS yielded values that were within a 2 fold range of published values, except that SV101 had a 7% P.E. instead of a 20% P. E., Ki Aγ4 had a 6% P. E. instead of a 20% P. E., and LS1 had a 0.01% P.E. instead of a 0.001% P.E. (Vogel and Pollack, 1974).

Growth Factors and Basal Medium

Insulin, Multiplication Stimulating Activity (MSA), EGF, and FGF (Fibroblast Growth Factor) were purchased from Collaborative Research. Human transferrin and dexamethasone were purchased from Sigma. The crude salivary gland extract ("gimmel factor") was prepared as described (Bottenstein et al., 1979) from rat submaxillary glands (Pel Freez, Arkansas). Fibronectin was purified from human plasma by gelatin affinity chromatography essentially as described (Engvall and Ruoslahti, 1977), with the modification that bound fibronectin was eluted with 1 M NaBr, pH 5.5. Fibronectin was dialyzed against PBS and diluted to 0.1 mg/ml or less before filter sterilization. Filtration resulted in a considerable loss of protein; hence the fibronectin concentration was determined after filtration. Partially purified PDGF was prepared from 200 units of outdated human platelets (New York Blood Center) by the method of Heldin et al. (Heldin et al., 1981a). Platelet lysate was purified sequentially by CM Sephadex chromatography, Blue Sepharose chromatography, and molecular sizing on a Bio Gel P150 column. The final steps were carried out aseptically in order to avoid filter sterilization. The

assay for PDGF mitogenic activity consisted of measuring the additional growth of 3T3 cells in totally defined serum free medium in the presence of insulin, EGF, and transferrin as described below. 60 ng/ml of the partially purified PDGF displayed mitogenic activity corresponding to 20 ng/ml purified PDGF (kindly provided by C. Heldin).

Serum Free Cell Culture

Two protocols were employed. The first protocol utilized serum coated dishes. Nunc tissue culture dishes were incubated with 10% serum /DME for 12 24 hrs., and then washed twice with serum free DME. Subconfluent dishes of cells grown in 10% serum were washed once with 0.5mM EDTA/PBS and allowed to detach in a small volume of PBS. The detached cells were diluted into MCDB 402 medium, centrifuged, resuspended and counted with the aid of a Coulter Counter. The cells were plated with sterile polypropylene pipet tips at various densities in MCDB 402 medium. Growth factors were added shortly afterwards with sterile polypropylene pipet tips. Plates were fed twice weekly. The growth factors used were insulin, 1-10 μg/ml as indicated; transferrin, 5 μg/ml; gimmel factor, 3-5 μg/ml as indicated; and FGF, 2-10 ng/ml as indicated.

The second protocol utilized culture dishes that were coated sequentially with sterile 0.1% poly D lysine (Sigma) for 10 minutes, washed once with PBS, and then treated with 1 μg/mm2 fibronectin in DME for 30 minutes at 37^{o} C, followed by one wash with serum free DME. Cells were harvested and plated in MCDB 402 medium as described above. Growth factors were added shortly afterwards: 10 μg/ml insulin, 40 ng/ml EGF, 0.1 μM dexamethasone, 10 ng/ml FGF, and 5 μg/ml transferrin. Experiments with PDGF replaced FGF with 60 ng/ml partially purified PDGF, and dexamethasone was deleted.

To determine the relative growth rates, cells were plated on 35 mm. or 60 mm. Nunc dishes as described in the text. Replicate plates for each condition were counted on the indicated days. The average number of doublings in the restrictive condition was divided by the average number of doublings in the nonrestrictive condition to determine the relative growth

rate. In some cases, the growth factor whose mitogenic response was being measured was not added until Day 1.

Relative plating efficiencies were determined by plating 10^3, 5 x 10^2, or 10^2 cells per 60 mm dish and allowing the cells to grow for 1 or 2 weeks as indicated in the text. Plates were fed two times each week, fixed in 10% formalin/PBS, and colonies were stained with either Giemsa or Crystal Violet stain. Depending on the duration of the experiment, colonies greater than either 0.5 mm or 1 mm, as indicated in the text, were scored as positive. Relative plating efficiencies were computed by dividing the average number of colonies in the restrictive condition by the average number of colonies in the non-restrictive condition.

The standard errors of relative measurements were determined by the relationship $(S_{rm})^2/(X_{rm})^2 = (S_r)^2/(X_r)^2 + (S_{nr})^2/(X_{nr})^2$ (Bennett and N.L., 1954), where X_{rm}, X_r, and X_{nr} are the average values of the relative measurement, growth in the restrictive condition, and growth in the non-restrictive condition, respectively, and S_{rm}, S_r, and S_{nr} are the corresponding standard errors. Analysis of variance was employed to determine the significance of the difference in relative measurements between two cell lines.

EGF Receptor Binding Assays

Specific binding of EGF receptors to cell surface receptors was determined as previously described (Fisher et al., 1980). Briefly, cells in the logarithmic phase of growth were seeded at various densities, depending on their growth rates, and binding assays were performed 48 to 72 hrs post plating. Two of the six replicate plates were used to determine the total cell number with the aid of a Coulter Counter. The remaining plates were washed twice with serum free DME before adding 2 ml binding buffer (DME:PBS, 2:1 (vol/vol) containing 1 mg/ml bovine serum albumin). Two plates were incubated with 50 µl of [^{125}I] EGF (specific activity 36 to 60 uCi/ug) and two plates were incubated with 50 µl [^{125}I] EGF and 500 ng/ml unlabeled EGF. Plates were incubated for 50 minutes at 37^o C in a 5% C02 humidified incubator. The monolayers were then rapidly washed 3X each with 5 ml cold assay buffer. The cells were then

solubilized for 2 hr at 37^{o} C in 1.5 ml of 0.85% Triton X 100, 0.02% EDTA and 0.25% trypsin in PBS. One ml aliquots were assayed for radioactivity in 10 ml of hydrofluor scintillation fluid. All of the receptor binding data are expressed as specific binding, i.e. the total binding obtained with labeled EGF minus the binding obtained in the presence of excess unlabeled EGF. The latter value represented < 5% of the total binding. Replicate plates agreed within 10%.

Results

The insulin growth requirement of Swiss 3T3

The starting point for our examination of the growth factor requirements of normal Swiss 3T3 cells and how they changed after transformation by SV40 was the growth factor formula for 3T3 developed by Serrero, Sato and McClure (1979). This formula includes insulin, fibroblast growth factor, transferrin, and a crude salivary gland extract termed gimmel factor. Provided that the culture dishes are pre coated with serum, and that the cells to be plated are gently harvested, this formula replaces serum for the growth of 3T3 cells (Serrero et al., 1979). To determine how to best assay for the insulin and FGF requirements, we varied two parameters: (1) the initial cell density and (2) the duration of the experiment. By measuring the doublings with and without a particular growth factor, we determined the relative growth rate (RGR), which reflects the stringency of the requirement for that particular growth factor.

We have found, as shown in Table 3-1, that the requirement for insulin is more stringent than the requirement for FGF (an average .28 RGR for insulin deprivation compared to an average .76 RGR for FGF deprivation). This ordering of the two requirements is in agreement with the findings of Serrero et al. (1979). The minor variations in the relative growth rates with FGF deprivation are not significant, regardless of the initial cell density or the duration of the experiment (Table 3-1). An altogether different phenomenon is apparent with insulin deprivation. A more stringent requirement for insulin was found when the cells were plated at

lower densities (Table 3-1). We also found that when 3T3 cells were plated at lower densities, the requirement for insulin was stronger when the culture period was extended from 4 days to 7 days (Table 3-1).

Table 3-1. Insulin and FGF Growth Requirements for Swiss 3T3 Plated at Monolayer Density

Inoculation Density	Days Grown	Doublings with All Growth Factors	Relative Growth Rate	
			(Ins/+Ins)	(FGF/+FGF)
1×10^5	4	2.3 ± .02	.48 ± .05	.74 ± .08
3×10^4	4	2.4 ± .02	.29 ± .02	.79 ± .07
1×10^4	4	2.5 ± .02	.32 ± .03	.80 ± .07
3×10^4	7	3.5 ± .02	.31 ± .02	.80 ± .03
1×10^4	7	3.9 ± .08	.15 ± .03	.72 ± .14
3×10^3	7	3.7 ± .02	.14 ± .02	.70 ± .09

Swiss 3T3 cells were plated at the indicated inoculation density onto serum coated dishes, with or without 10 μg/ml insulin, with or without 10 ng/ml FGF, plus 5 μg/ml gimmel factor and 5 μg/ml transferrin. Doublings, relative growth rate, and the standard error (n = 3) were determined as outlined in Experimental Procedures. Analysis of variance showed that the effect of cell density was significant for insulin deprivation ($p < .01$ that cell density had no effect) but not significant for FGF deprivation ($p > .50$).

The ability to accentuate the requirement for insulin by plating cells at lower densities and lengthening the culture period led us to attempt growing 3T3 at colony forming densities. In preliminary experiments, we found that the basal medium comprised of a mixture of DME and F12 used by Serrero et al. (1979) with added growth factors did not support the clonal growth of 3T3. However, the mouse fibroblast medium MCDB 492 developed by Shipley and Ham (Shipley and Ham, 1981) did support clonal growth. In addition to the requirement for an adequate basal medium, Swiss 3T3 displayed a very strong requirement for insulin in order to efficiently form colonies (Table 3-2). As was found in the relative growth rate assay, the requirement for FGF was more moderate than the requirement for insulin in the relative plating efficiency assay (Table 3-2). Again, no effect of cell density was observed for the requirement for FGF, but there was significant density dependence for the insulin requirement (Table 3-2). Thus, the three phenomena observed in the relative growth rate assay were also found in the relative plating efficiency assay, namely: (1) a more stringent requirement for insulin than for

FGF (2) a lack of density effect for the FGF requirement and (3) a noticeable density effect for the insulin requirement. Perhaps the most straightforward explanation for this latter phenomenon is that 3T3 produces endogenous insulin like growth factor that would be diluted at lower densities.

Table 3-2. Insulin and FGF Growth Requirements for Swiss 3T3 Plated at Colony Forming Density

Inoculation Density	Plating Efficiency in Full Supplement	Relative Plating Efficiency	
		(Ins/+Ins)	(FGF/+FGF)
3×10^3	12 ± 1	.10 ± .01	.83 ± .12
1×10^3	16 ± 1	.04 ± .01	.75 ± .06
3×10^2	27 ± 4	.004 ± .008	.52 ± .23

Swiss 3T3 cells were plated at the indicated inoculation densities on 60 man. dishes as described in the legend to Table 1. 10 days later, colonies greater than 0.5 mm in diameter were scored. The lower efficiency of colony formation with the full supplement at the higher inoculation densities is also observed in 10% FCS, and reflects the non linearity of the colony formation assay as the upper limit for colonial growth is approached (about 104 cells for 60 mm. dishes). Relative plating efficiencies and the standard error (n = 3) were determined as outlined in Experimental Procedures. Analysis of variance showed that the effect of cell density was highly significant for insulin deprivation ($p < .01$) but less significant for FGF deprivation ($p < .10$).

The Effect of SV40 Transformation on the Growth Requirement for Insulin

Previously, other investigators have reported that SV40¬transformed cells require insulin for growth in serum free medium (Rockwell et al., 1980; Young and Dean, 1980). However, no comparison was drawn with normal cells. As seen in Table 3-3, the strong insulin requirement of normal 3T3 is severely diminished in SV101, both in the relative plating efficiency assay and in the relative growth rate assay. Since SV101 mitogenically responds to insulin, we agree that SV40 transformed 3T3 cells require insulin for optimal growth. However, when the response or requirement is compared with normal 3T3, it is apparent that SV101 does not slow down its growth in response to insulin deprivation to nearly the same extent as does normal 3T3 (Table 3-3).

Table 3-3. The Insulin Growth Requirement of Swiss 3T3, SV40 transformed 3T3 and Revertants

Cell Line	Relative Plating Efficiency		Relative Growth Rate
	Expt. 1	Expt. 2	
Swiss 3T3	.01 ± .01	.05 ± .02	.34 ± .01
SV101	.34 ± .08	.47 ± .11	.87 ± .02
AγSV4	.01 ± .01	.03 ± .02	.39 ± .03
LSSVl	.06 ± .05	nd	nd

In Expt 1, 500 cells (for 3T3 and AγSV4) or 100 cells (for SV101 and LSSV1) were plated in 10% FCS/DME, and 24 hours later the plates were washed twice with serum-free DME before adding MCDB 402 with or without 1 μg/ml insulin, plus 2 ng/ml FGF, 3 μg/ml gimmel factor, and 5 μg/ml transferrin. 10 to 14 days later, duplicate plates for each condition were fixed and scored for colonies greater than 1 mm in diameter. 3T3 and AγSV4 formed no colonies without insulin when 100 cells were plated. In Expt. 2, 1000 cells were plated onto serurrrcoated dishes as described in the legend to Table 1. 7 days later, duplicate plates for each condition were scored for colonies greater than 0.5 mm in diameter. "The relative growth rate was determined by plating 10,000 cells onto 60 mm dishes and measuring the doublings after 4 days growth as described in the legend to Table 1.

Previously, we had isolated revertants of SV101 that had regained a high serum growth requirement. One of these revertants, LS1, was negatively selected by culturing SV101 in 1% calf serum in the presence of the killing agent BUdR. LS1, like normal 3T3, grows poorly in 1% serum (Vogel and Pollack, 1973). As seen in Table 3-3, LS1 did not form colonies efficiently in the absence of insulin, paralleling its reacquisition of a normal serum requirement. The serum sensitive revertant Aγ4 also formed colonies inefficiently without insulin (Table 3-3). In the relative growth rate assay, it resembled normal 3T3 in terms of its response to insulin deprivation (Table 3-4). Thus, both serum sensitive revertants of SVI01 were found to have regained a 3T3 like insulin requirement.

Since these results were obtained with partially defined medium it might be argued that, in the complete absence of any insulin like molecules that may well be contained in the crude salivary gland extract or absorbed onto the dish during the serum coating, SV101 would display the same strong insulin requirement observed in normal 3T3 and the serum sensitive revertants. To test this possibility, we developed totally defined conditions for the growth of Swiss 3T3. This involved providing an adequate substrate to replace the serum coating. After testing

various combinations of collagen, poly-D-lysine, and fibronectin coating, we settled upon the sequential treatment of dishes with 0.1% poly D lysine followed by treatment with fibronectin (Wolfe et al., 1980). This method was superior to any other tested for the growth of 3T3 cells. Additionally, we substituted the known 3T3 mitogens EGF and dexamethasone (Carpenter and Cohen, 1979; Thrash and Cunningham, 1974) for the crude salivary gland extract.

Table 3-4. Insulin Growth Requirement of Mouse Fibroblasts in a Totally Defined Medium

	Growth Control Phenotypes			Relative Growth Rate
Cell line	**Serum**	**Density**	**Anchorage**	**-Insulin /+Insulin**
Swiss 3T3	N	N	N	.30 ± .06
SV101	TX	TX	TX	.73 ± .05
FLSV	TX	N	N	.79 ± .07
LSSVI	N	N	N	.53 ± .02
AγSV4	N	N	TX	.10 ± .04

Cells were plated at a density of 2 x 10^4 / 35 mm dish. The dishes were coated with poly D lysine/fibronectin. The medium was MCDB 402 supplemented with 40 ng/ml EGF, 10 ng/ml EGF, 1 x 10^{-7} M dexamethasone, and 5 μg/ml transferrin. Some plates received insulin. Duplicate plates for each condition were counted on day 1 and day 5. The numbers were converted to c'bublings and the values represent the relative growth rates ± standard errors.

SV101 did not display a strong insulin requirement in the totally defined medium, when compared to the insulin dependence of normal 3T3. The reacquisition of a stronger insulin requirement we observed previously for the serum sensitive revertants holds true in this totally defined medium, although LSSV1 displayed a more moderate insulin requirement than 3T3 (Table 3-4). However, LSSVl is significantly more insulin dependent than SV101 (Table 3-4). FLSV, a flat anchorage dependent revertant of SV101 that retained a low serum requirement (Vogel and Pollack 1973), displayed a minimal requirement for insulin, similar to the minimal requirement of SV101. This shows that the loss of the insulin requirement co segregates with the loss of serum dependence and not with the loss of the anchorage requirement or the loss of density dependent growth inhibition. The lower relative growth rates obtained in this

experiment, compared with the values in Table 3-3, indicate that the totally defined medium provides a more stringent test of the insulin requirement than the partially defined medium.

Retransformation of the Revertant Aγ4 Does Not Reduce its Insulin Requirement

Both LS1 and Aγ4 were found to retain SV40 nuclear T-antigen (Vogel and Pollack, 1973). We have also found that these two cell lines, like SV101, express normal levels of both 94K T-antigen and 17K t-antigen (Chen et al., 1981), even after growth in 1% calf serum (unpublished experiments). Were the reacquisition of a strong insulin requirement to be ascribed to subtle damage to the structure of these SV40 T-antigens, it ought to be possible to retransform these cells, using either SV40 or another tumor virus that can reduce the normal insulin requirement. We have found that Kirsten Murine Sarcoma Virus (Ki-MuSV) transformed 3T3 have a significantly reduced insulin requirement (Table 3-5). Therefore, we examined the insulin requirement of a Ki-MuSV transformed derivative of Aγ4 (Ki-Aγ4).

Table 3-5. The Effect of Kirsten MuSV Transformation on the Insulin Growth Requirement

	Growth Control Phenotypes			Relative Growth Rate
Cell Line	**Serum**	**Density**	**Anchorage**	**(-Ins/+Ins)**
NIH 3T3	nd	nd	N	.36 ± .03
NIH DT	nd	TX	TX	.98 ± .02
Ki-Aγ4	N	TX	TX	.19 ± .05

Conditions were exactly as described in the legend to Table 3-4. nd = not determined.

Ki-MuSV transformation converted the flat morphology of Aγ4 into the typical spindly morphology of Ki-MuSV transformed cells, and furthermore it obviated the density dependent growth inhibition of Aγ4 (Vogel and Pollack, 1974). This indicates that Ki-Aγ4 most likely expresses the K-ras p21 transforming protein (Furth et al., 1982). However, as seen in Table 3-5, Ki-Aγ4 retained a strong requirement for insulin, which argues against the notion that Aγ4 has a high insulin requirement because of mutated SV40 gene products.

It appears, then, that Ki Aγ4 expresses both SV40 and Ki MuSV transforming gene products, yet remains normal with respect to its insulin requirement. A simple explanation for these results is that Av4 has suffered a cellular mutation that affects the mechanism by which both SV40 and Ki MuSV transforming gene products overcome the normal insulin requirement.

Diminished EGF Requirement in SV40 Transformed 3T3 Cells

The ability to leave out the crude salivary gland extract, which contains contaminating EGF (McClure et al., 1981), led us to examine the effect of EGF deprivation on normal 3T3 cells. The requirement for EGF was more moderate than the requirement for insulin (.30 RGR for insulin deprivation compared with a .68 RGR for EGF deprivation). The EGF requirement of SV101 was significantly lower than the EGF requirement of 3T3 (Table 3-6). Interestingly, the EGF requirement of the serum sensitive revertant Aγ4 remained diminished, although its insulin requirement was very strong. Thus, Aγ4 appears to have reacquired sensitivity to serum as a result of its specific reacquisition of a strong insulin requirement, without a concomitant reacquisition of a 3T3 like EGF requirement.

Table 3-6. The EGF Requirement of Mouse Fibroblasts

Cell Line	Relative Growth Rate (-EGF/+EGF)
Swiss 3T3	.68 ± .11
SV101	.85 ± .05
FLSV	1.05 ± .04
Aγ4	.92 ± .11
Ki-Aγ4	1.08 ± .05
LS1	.66 ± .03

The conditions were exactly as described in the legend to Table 3-4, except that EGF was left out or put in in place of insulin.

The serum sensitive revertant LS1, on the other hand, was found to have reacquired a 3T3 like EGF requirement (Table 3-6). LS1, unlike Aγ4, displayed a more moderate insulin requirement than 3T3 (Table 3-4), and may have reacquired sensitivity to serum as a result of reacquiring both a moderate insulin requirement and a 3T3 like EGF requirement. It should be noted that LS1 was isolated by negative selection under conditions of reduced serum concentrations (1%), whereas Aγ4 was isolated by negative selection under conditions of chemical and biological removal of certain serum fractions (Vogel and Pollack, 1973). It is possible, therefore, that the different selective pressures utilized to isolated serum sensitive revertants, i.e. low serum versus "growth factor depleted" serum, results in the generation of two distinct classes of serum sensitive revertants.

FLSV, the flat, anchorage dependent but serum transformed revertant of SV101, had no requirement for EGF, indicating that the loss of the EGF requirement is not necessarily linked to the loss of the anchorage requirement or the loss of density dependent growth inhibition. This is in agreement with a recent report that the loss of the EGF requirement is dissociable from anchorage independence (Sager et al., 1982).

Selectivity of the PDGF Requirement

The doubling time of 3T3 cells in the fully supplemented, totally defined medium was 48 hours, compared to a 23 hour doubling time in 10% FCS. In contrast, SV101 had a 19 hour doubling time in the fully defined medium, compared to a 16 hour doubling time in 10% FCS. This represented a 1.1 fold slower growth rate for 3T3 but only a 0.3 fold slower growth rate for SV101. This discrepancy in the relative effectiveness of the defined medium implied that the medium lacked a serum growth factor that was required by 3T3 more than it was required by SV101. Although FGF is as potent as PDGF in stimulating one round of DNA synthesis in confluent 3T3 cells (Stiles et al., 1979), we found that both partially purified PDGF and purified PDGF (kindly provided by C. Heldin) were more stimulatory than FGF for the continuous growth of 3T3 cells in the totally defined medium. The doubling time for Swiss 3T3

with PDGF in place of FGF was 24 hours, compared to the 48 hour doubling time observed with FGF. SV101 did not respond at all to PDGF, doubling once every 19 hours, with or without PDGF, indicating that SV40 transformation can totally abolish the requirement for PDGF.

Since we had previously observed a reacquisition of the insulin requirement in serum sensitive revertants, we determined whether they also reacquired a PDGF response. Both serum sensitive revertants regained 3T3 like PDGF requirement. However, the flat revertant FLSV regained an even stronger PDGF requirement although FLSV remained serum transformed Table 3-7). Since both serum sensitive revertants, like FLSV, regained density dependent growth inhibition, it is possible that the reacquisition of a PDGF requirement is related to this particular growth control phenotype. In support of this notion, Ki-Aγ4 was found to have lost the PDGF requirement of Aγ4, concomitant with its loss of density dependent growth inhibition. Since Ki Aγ4 retained a normal serum requirement, our results indicate that the loss of the PDGF requirement does not in itself confer reduced serum dependence. Rather, the loss of the PDGF requirement appears to be related to the loss of density dependent growth inhibition.

Table 3-7. The PDGF Requirement of Mouse Fibroblasts

Cell Line	Growth Control Phenotypes			Relative Growth Rate
	Serum	Density	Anchorage	(PDGF/+PDGF)
Swiss 3T3	N	N	N	0.52
SV101	TX	TX	TX	1.04
FLSV	TXI	N	N	0.22
Aγ4	N	N	TX	0.48
Ki-Aγ4	N	TX	TX	1.01
LSl	N	N	N	0.62

The PDGF requirement was determined by plating 2 x 10^4 cells / 35 mm dish on poly-D-lysine/fibronectin coated dishes containing 2 ml MCDB 402 plus 10 μg/ml insulin, 5 μg/ml transferrin, and 40 ng/ml EGF. 24 hours later, duplicate plates were counted and 60 ng/ml partially purified PDGF was added to half of the remaining dishes. Cell counts were determined 4 days later.

EGF receptors are reduced in serum sensitive revertants

Both the reduced EGF requirement of Ki MuSV transformed cells and their ability to grow in soft agar has been ascribed to their production of a transforming growth factor, termed sarcoma growth factor, which binds to the EGF receptor and prevents the binding of exogenous labeled EGF (De Larco and Todaro, 1978; Kaplan et al., 1981). Also, we have recently found that adenovirus transformed rat cells secrete a polypeptide growth factor that binds to the EGF receptor and induces normal rat cells to grow in soft agar (Fisher et al., 1983). In light of these findings, we were interested in determining whether the loss or the reacquisition of growth factor requirements were accompanied by changes in the corresponding cell surface receptors. As discussed beforehand, dose response studies with insulin and the insulin like growth factor MSA indicated that one of the IGF receptors and not the high affinity insulin receptor was mediating the growth effects we have observed. Unfortunately, we are not in a position to purify either IGF or PDGF to homogeneity, precluding us from examining these particular receptors. However, the ready availability of highly purified EGF enabled us to examine the EGF receptors in this set of cell lines. Although SV101 had a diminished EGF requirement, we found that SV101 bound only slightly lower levels of EGF than the parental cell line, Swiss 3T3 (Table 3-8). This finding is in agreement with previous studies which indicated that SV40 or polyoma virus transformation is not associated with major alterations in EGF receptor binding (Todaro et al., 1976). In the case of the flat revertant FLSV, which also displayed a reduced EGF requirement, the degree of EGF binding was similar to control 3T3 cells (Table 3-6). Apparently, the reduced EGF requirement observed in SV40 transformation is not mediated by the production of an EGF like growth factor. In accordance with the findings of Todaro et al. (1976) we found that the Ki MuSV transformed derivative of Aγ4 had negligible amounts of EGF binding (Table 3-8).

In contrast to the results we obtained with 3T3, SV101, and FLSV, we found that the serum sensitive revertant LS1 had significantly reduced amounts of EGF receptor binding (Table 3-8). Since LS1 had regained a 3T3 like EGF requirement, it is unlikely that the

Table 3-8. EGF receptor binding to SV40 3T3 and revertants

Cell line	EGF binding - cpm/10^6 cells	
	Confluent	Subconfluent
Swiss 3T3	(100)	(100)
SV101	88 ± 2	74 ± 2
FLSV	102 ± 2	109 ± 2
LSSV1	20 ± 2	39 ± 5
AγSV4	46 ± 10	53 ± 1
Ki-Aγ4	4 ± 2	8 ± 2

EGF binding was determined as described in Materials and Methods.

reduction in EGF binding is due to the moderate secretion of an EGF like growth factor. In the case of Aγ4, a more modest reduction in EGF binding was observed (Table 3-8), but this reduction was not associated with a reacquisition of a 3T3 like EGF requirement. As previously indicated, the different selective pressures used to generate the serum sensitive revertants LS1 and Aγ4 may have resulted in two distinct classes of serum revenants, i.e. some with a more modest insulin requirement and a 3T3 like EGF requirements (LS1) and others with a very strong insulin requirement but a reduced EGF requirement (Aγ4 and Ki-Aγ4). In the case of LSl, serum dependence may in part have resulted from an alteration in the EGF receptor system that resulted in the reacquisition of a stronger EGF requirement. This would imply that the reduction in EGF receptors found in Aγ4 was the result of some other alteration not associated with the reacquisition of serum dependence. Alternatively, the reduction of EGF binding in the serum sensitive revertants may be secondarily related to serum dependence. Robinson et al. (Robinson et al., 1982) found a cell cycle dependence of EGF binding in chemically transformed mouse fibroblasts. In unpublished experiments, we found that the cell cycle distribution of the serum sensitive revertants differs from SV101 when they were grown in 10% FCS, and this may underlie the observations we have made.

Discussion

Our results indicate that SV40 transformation can drastically alter the growth factor requirements of normal fibroblasts. All three of the growth factor requirements found in normal 3T3 cells were diminished or abolished following SV40 transformation. This multiple loss of growth factor requirements is not unique to SV40 transformation. We have shown that Kirsten MSV transformation also reduced both the insulin and PDGF requirements, and others have shown that Kirsten MSV transformation reduces the EGF requirement (Kaplan et al. 1982).

It will be important to determine whether any or all of these diminished growth factor requirements are necessary for tumor formation. Neoplastic transformation can alter many cellular properties, but only some of them have been shown to be directly related to tumorigenicity. For example, the loss of cell surface fibronectin is not necessary for tumor formation (Kahn and Shin, 1979). On the other hand, it has been well established that anchorage independence is a necessary prerequisite for tumor formation of both fibroblastic and epithelial cells (Kahn and Shin, 1979). Recently, Perez-Rodriguez et al. (Perez-Rodriguez et al., 1981) reported that a reduced serum requirement, in addition to anchorage independence, was a necessary prerequisite for tumorigenicity in hamster fibroblasts.

Many transformed cells and human tumor cell lines would appear to be able to escape the requirement for specific growth factors by virtue of their secretion of growth factors homologous to either EGF or the insulin like growth factor MSA (Todaro et al. 1979). Murine sarcoma virus and adenovirus transformed rodent cells secrete an EGF-like growth factor that induces certain normal rat cell lines to grow in soft agar, and also prevents the binding of exogenous labeled EGF (DeLarco and Todaro, 1978; Fisher et al. 1983). While secretion of an EGF like growth factor appears to be the mechanism operative in the reduced EGF requirement of Kirsten MSV transformed fibroblasts (Kaplan et al. 1982), we have found that SV40 can diminish the EGF requirement without causing a significant reduction in EGF binding. In contrast, serum sensitive revertants had significantly diminished EGF binding, but the meaning of this result is not clear, since only one of the serum sensitive revertants regained a 3T3 like

EGF requirement. However, the fact that only serum sensitive revertants displayed reduced EGF binding would seem to indicate that alterations in the EGF receptor system are involved in reversion to serum dependence.

Two significant findings emerged from the selective reacquisition of growth factor requirements by revertants: (1) the loss of the PDGF requirement was directly associated with the loss of density dependent growth inhibition, and (2) the loss of the strong insulin requirement was directly associated with a reduced serum dependence. In light of the first finding, it has been known for some time that the final saturation density of normal fibroblasts is directly proportional to the serum concentration (Holley and Kiernan, 1968). Recently, Vogel et al. (Vogel et al., 1980) have provided evidence that PDGF is the serum component responsible for the determination of final saturation density. Our results are in concert with this finding, and suggest that the loss of the PDGF requirement enables cells to grow to high saturation densities. Selective reacquisition of only the PDGF requirement appears to be a sufficient alteration to restore density dependent growth inhibition to the flat revertant FLSV.

Our finding that the loss of a stringent insulin requirement is directly related to a reduced serum dependence is in agreement with the work of Temin, who found that Rous sarcoma virus transformed chick fibroblasts had a reduced serum requirement (Temin 1966), and that this reduced serum dependence resulted from a diminished requirement for the insulin replaceable activity in serum (Temin 1967). Partial purification of the major mitogenic activity in calf serum for the growth of chicken fibroblasts revealed that this activity was closely related to the insulin like growth factor MSA (Dulak and Temin, 1973; Pierson and Temin, 1972). Other laboratories have reported a reduced insulin requirement following neoplastic transformation. Moloney sarcoma virus transformed dog kidney epithelial cells and spontaneous tumors of mouse mammary epithelium have been reported to have a reduced requirement for insulin when compared with their normal counterparts (Imagawa et al., 1982; Taub et al., 1981). As discussed previously, the insulin growth requirement, at least for fibroblasts, is likely to represent a requirement for somatomedin or insulin like growth factor

(King and Kahn, 1981). Somatomedins are thought to mediate many if not all of the growth promoting effects of growth hormone (Daughaday et al., 1975; Van Wyk and Underwood, 1975). It would not be surprising if escape from this major humoral growth regulatory system by a single cell influenced its growth potential *in vivo*.

Many growth control revertants result from changes in the transforming gene itself, either through total deletion (Steinberg et al., 1978), mutation (Varmus et al., 1981)(Varmus et al., 1981), transcriptional inactivity (Porzig et al., 1979), or selective loss of integrated viral DNA (Blanck et al., 1983). While these revertants have provided solid evidence in favor of the hypothesis that continued transforming gene expression is required for the maintenance of the transformed state, revertants that result from second site cellular mutations offer the possibility of identifying cellular molecules involved in growth control. We have shown that the strong insulin requirement of the serum sensitive revertant AγSV4 cannot be altered by Kirsten MSV transformation, although Kirsten MSV transformation reduced the insulin requirement of normal 3T3 cells. We are performing cell hybridizations to determine whether the strong insulin requirement of the revertant AγSV4 behaves as a dominant or recessive mutation. If it is recessive, it may be possible to identify the mutated gene by gene transfer.

Addendum to Chapter Three

The following results were not analyzed in time to be integrated into Chapter 3. In order to extend the results described in the previous chapter, I set up a one week growth assay, using the newly developed totally defined medium with PDGF. As mentioned in Chapter 1, PDGF stimulates the production of IGF-I and thereby lowers the insulin requirement. Nevertheless, I felt it was important to determine whether or not SV40-transformed 3T3 would display a lower insulin requirement than 3T3 in the presence of PDGF. Although the difference is small (Table 3-9), it is statistically significant ($p < .01$). In addition, I wanted to repeat the results obtained for the EGF and the insulin requirements with this particular set of cells, in the presence of PDGF. Unfortunately, in terms of the PDGF requirement, this experiment was set up differently from the previous ones, i.e. the cells were plated at a 5-fold lower inoculation density in order to enhance the growth response. This seemingly small variation in inoculation density effects the PDGF requirement significantly, as will be discussed in Chapter 6. Nevertheless, both 3T3 and the revertants showed a significant PDGF requirement, and the small difference in the PDGF requirement of Aγ4 and KiAγ4 in this experiment (Table 3-9) is statistically significant by the F-test ($p < .05$). 3T3 did not display a significant EGF requirement at all in the presence of PDGF. It should be noted that other laboratories working on the serum free growth of Balb/3T3 do not see a significant response to EGF (McClure, Hightower and Topp, 1982). The only cell line that did show a large response to EGF in this experiment was Aγ4 (Table 3-9). I realize that this result is totally at odds with the findings discussed in Chapter 3. Unfortunately, I have no explanation for this variable result. PDGF is known to effect the processing of EGF receptors, and perhaps this underlies the difference (Wrann et. al., 1988).

Table 3-9. Insulin, EGF, and PDGF Requirements of Mouse Fibroblasts

Cell Line	Doubling Time (hrs)	Relative Growth Rate		
		-Ins	-EGF	-PDGF
Swiss 3T3	25	0.71	0.96	0.56
SV101	18	0.83	0.93	1.00
Aγ4	31	0.31	0.66	0.92
Ki-Aγ4	24	0.50	0.99	1.06
LS1	32	0.60	0.94	0.77
FLSV	24	0.75	0.89	0.67

Cells were plated at a density of 10^4 cells / 60 mm dish in MCDB 402 with 5 μg/ml transferrin. 2 hours later, duplicate plates were counted and the appropiate growth factors were added to the remaining plates: PDGF, 60 ng/ml; insulin, 10 μg/ml; and EGF, 40 ng/ml. The plates were fed on day 4 and counted on day 7. The doublings were converted to doubling time and the relative growth rates were determined as described in the Materials and Methods section of Chapter 3.

CHAPTER 4

ENDOCYTOSIS

This work was done in collaboration with Robert F. Murphy

Abstract

Neoplastic transformation often results in the loss of growth control and concomitant changes in cell surface properties. The changes in endocytosis of a variety of probes after transformation were measured for mouse fibroblasts by flow cytometry. No major differences in dextran (fluid phase) or histone (non specific adsorptive) endocytosis were observed among four cell lines having different growth properties. However, decreased receptor mediated internalization of $\alpha 2$ macroglobulin was observed for transformed cell lines. Furthermore, increased wheat germ agglutinin endocytosis and decreased insulin endocytosis were also observed in transformants. These changes were not accounted for by changes in wheat germ agglutinin or insulin binding.

Introduction

Many cellular changes have been reported to accompany the conversion of a normal cell to a neoplastic cell. Transformation of fibroblasts by agents such as SV40 can result in the elimination of many growth requirements, such as those for anchorage, and serum (Risser and Pollack, 1974). Changes in the cell surface and the cytoskeleton have been of particular interest because of their proposed roles in controlling cellular growth (Pollack et al., 1975). Unfortunately, it has not been possible to develop these observations into an explanation for the mechanism that underlies the ability of a transformed cell to grow under restrictive conditions. For example, the alterations in nutrient transport which have been observed may be secondary to the growth state of the cell and not intrinsically different in normal and transformed cells

(Dubrow et al., 1978). However, it is reasonable to expect that certain molecular constituents of the cell surface or the cytoskeleton will be of primary importance in the loss of growth control.

Endocytosis is a cellular function that involves both the cell surface and the cytoskeleton. Cytochalasins have been shown to inhibit endocytosis in a variety of systems (Salisbury et al., 1980). Endocytosis has been suggested to play a role in mitogenic stimulation by growth factors (Goldfine, 1981), but quantitative comparisons of fluid phase and receptor mediated endocytosis by normal and transformed cells have not previously been reported. We have therefore begun an analysis of the changes in endocytosis that accompany transformation to either serum or anchorage independence. We have used flow cytometry to analyze the binding and endocytosis of various fluorescent probes by mouse fibroblast cell lines. Flow cytometry allows the rapid and accurate quantitation of fluorescent and scattered light resulting from the passage of individual cells through a focused laser beam.

Materials and Methods

Cell Culture

Cell lines were maintained in DME containing 10% (vol/vol) FCS with 100 μg/ml streptomycin and 100 units/ml penicillin. The serum requirement was determined by plating approximately 1000 cells/cm2 in both 1% and 10% calf serum and counting duplicate dishes on day 1 and day 4. The number of doublings in 1% serum was divided by the number of doublings in 10% serum to yield the relative growth rate (RGR). The anchorage requirement was determined by plating cells in 3 ml 1.3% methocel medium over a 2 ml 0.5% agarose underlay in 60 mm dishes. Plates were fed weekly with 5 ml methocel medium and scored for colonies greater than 0.2 mm in diameter after 3 weeks of incubation. The number of such colonies was divided by the number of cells plated to yield colony forming efficiency (CFE).

Incubation of cells with fluorescent probes

The preparation and properties of the fluorescein isothiocyanate_(FITC) conjugated insulin have been previously described (Murphy et al., 1982b). The FITC insulin had 56% of the activity of unlabeled_insulin for stimulating DNA synthesis. 5-iodoacetamido fluorescein (IAAF) conjugated histone was prepared as described (Murphy et al., 1982a). FITC dextran and FITC wheat germ agglutinin were obtained from Sigma. FITC α-2M was the generous gift of Dr. F. Maxfield.

For binding measurements, subconfluent monolayers were washed once with cold phosphate buffered saline (PBS), and then incubated for 60 minutes at 40 C with 0.1 μM FITC WGA or 1 μM FITC insulin in PBS with 1 mM $CaC1_2$ and 0.5 mM $MgC1_2$. The cells were harvested by washing once with PBS and detaching the cells with 0.5 mM EDTA/PBS. An equal volume of 10%FCS/PBS was added and the suspended cells were kept on ice until analysis to prevent internalization of bound probe.

For measurements of the endocytosis of FITC insulin, FITC WGA, FITC dextran and IAAF histone, subconfluent cultures (60 mm dishes) were incubated with probes (added directly to the growth medium) for 150 min at 37^{o} C. The final concentration of the probes were: FITC-insulin, 1 μM; FITC WGA, 0.1 μM; FITC dextran, 1 μM; IAAF histone, 0.78 μM. Subconfluent cultures which had been washed twice with DME were incubated for 30 minutes at 37^{o} C with 80 μg/ml FITC α2M in serum free DME. For all probes, plates were washed once with 0.0025% trypsin/0.5 mM EDTA/PBS, and cells were detached by incubating for 10 minutes in 1 ml of the same solution. 1 ml of 10%FCS/0.05%NaN3/PBS was added and the suspended cells were kept on ice until analysis.

Fluorescence Measurements

Mean fluorescence per cell was determined using a FACS IV (Becton Dickinson, Mountain View, CA). Excitation was with the 488 nm line of an argon ion laser, and emission was measured using a 520 nm long pass dielectric filter and a 520 nm long pass optical glass filter. Sample temperature was maintained at 4^{o} C by a circulating water bath containing

ethylene glycol/water. Fluorescence measurements were converted to number of molecules per cell using fluorescent bead standards (Murphy et al., 1982b) and were corrected for autofluorescence of unlabeled cells. Cell volumes were measured using a Coulter counter equipped with a model P64 channel analyzer.

Results

To study the effect of transformation on endocytosis in general, we used four cell lines having different properties with respect to their insulin and serum requirements (Table 4-1).

Table 4-1. Properties of the Four Cell Lines

Cell Line	Growth Requirements		Cell Volume	Surface Area	Light Scattering
	Serum	Anchorage			
	(RGR)	(CFE)	(m^3)	(m^2)	
3T3	0.26	< 0.001	2660	928	69
Aγ4	0.02	1	3070	1020	73
SV101	0.67	7	2140	803	56
3T6	0.65	< 0.001	2370	858	63

The average standard errors of the mean for the cell volume, surface area and light scattering measurements were 2%, 1.3%, and 8.3%, respectively.

We used three fluorescent probes that are internalized by different mechanisms. FITC dextran is internalized by fluid phase pinocytosis (without binding to the cell surface (Berlin and Oliver, 1980)). IAAF histone is internalized by non-specific adsorptive endocytosis (Murphy et al., 1982b). FITC α2 macroglobulin is a protease inhibitor found in serum, which undergoes receptor mediated endocytosis. We measured the internalization of these probes by the four cell lines using flow cytometry. An example of the results of this analysis for α2-M internalization by 3T3 and SV101 are shown in Figure 4-1. The high degree of specificity of the internalization is shown by the effect of the addition of an excess of unlabeled α2 M. For both cell lines, the internalization is 92% specific. SV101 specifically endocytosed 38 ± 5% less α2-M than 3T3.

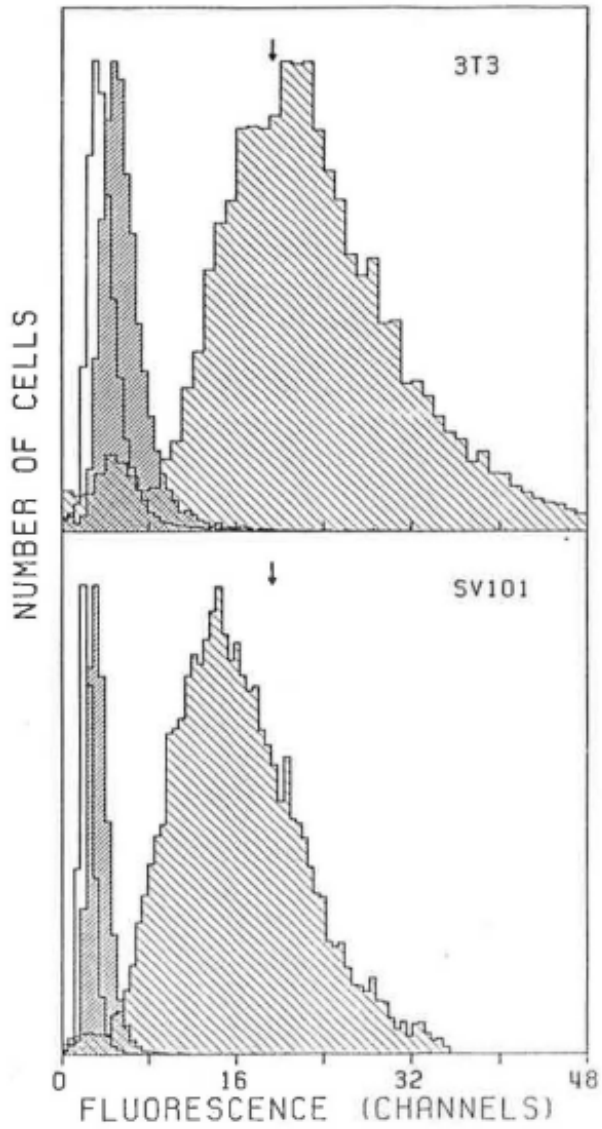

Figure 4-1. Receptor mediated endocytosis of α2-macroglobulin by normal and SV40-transformed fibroblasts. Monolayers were incubated with 80 ug/ml FITC-α2-M for 30 min at 370 C in the absence (\\\) or presence (///) of 3 mg/ml unlabeled α2-M, trypsinized, and then analyzed by flow cytometry in the presence of 100 μM chloroquine (to prevent quenching of FITC by low pH.) Histograms for unlabeled cells (unshaded) and the position of fluorescent bead standards (arrow) are also shown.

The results of similar measurements for the other cell lines and probes are shown in Table 4-2. No major differences in the amount of FITC dextran or IAAF histone endocytosis were observed, especially when these values were normalized for surface area (using the data in

Table 4-1). We conclude that neoplastic transformation need not impair the mechanisms involved in these bulk endocytic processes but may affect receptor mediated endocytosis.

Table 4-2. Comparison of Different Types of Endocytosis in Normal and Transformed Fibroblasts

Cell Line	FITC-Dextran		IAAF-Histone		α2-macroglobulin		
	Total		Total		Total	Specific	
	Per Cell	Surface Area	Per Cell	Surface Area	Per Cell	Per Cell	Surface Area
3T3	0.385	0.42	163	176	21.5	17.5	18.9
Aγ4	0.361	0.35	180	176	11.6	6.2	6.1
SV101	0.28	0.35	189	235	14.1	10.4	12.9
3T6	0.232	0.27	147	171	9.7	5.3	6.2

See Materials and Methods for concentrations and conditions. The standard errors of the mean for the dextran and α2-M internalization measurements were 8.4% and 9.0%, respectively.

In contrast to the results for α2-M, SV101 and 3T6 endocytosed more FITC wheat germ agglutinin (FITC-WGA) than 3T3 or Aγ4 (Table 4-3). This difference is not explained by differences in the amount of lectin bound per cell. Based on previous results which demonstrated that increased agglutinability after transformation resulted not from changes in the number of lectin binding sites but from changes in the mobility of these sites (Burger, 1969), we interpret our results to mean that increased lectin site mobility contributes to increased lectin endocytosis. Compared to SV101, Aγ4 endocytosed less α2-M, despite increased binding. The rate of internalization of lectin sites is therefore even lower for Aγ4 than for 3T3, despite the fact that Aγ4 grows well in suspension (Table 4-1) and has a disorganized actin cytoskeletal system typical of transformed fibroblasts (Pollack et al., 1975). The higher rate of WGA endocytosis in SV101 and 3T6 is therefore likely to be directly related to serum transformation, and unrelated to those actin cytoskeletal changes that can be detected at the level of light microscopy.

Table 4-3. Endocytosis and Binding of Wheat-Germ Agglutinin and Insulin

Cell Line	FITC-WGA			FITC-Insulin			
	Binding	Endocytosis		Binding		Endocytosis	
	Per Cell	Per Cell	Surface Area	Per Cell	Surface Area	Per Cell	Surface Area
3T3	0.47	3.3	3.6	1.5	0.74	4.0	4.3
Aγ4	0.72	4.1	4.0	2.0	0.45	3.8	3.7
SV101	0.58	5.3	6.7	1.5	0.39	2.1	2.6
3T6	0.35	5.4	6.3	2.0	0.48	2.3	2.7

See Materials and Methods for concentrations and conditions. The average standard errors of the mean for the WGA and insulin endocytosis measurements were 8.3% and 9.9% respectively.

Table 4-3 also show the results of measurements of insulin binding and endocytosis. For these measurements, we used the supraphysiological concentration of insulin needed to stimulate cell growth, as discussed below. Compared with WGA endocytosis, the behavior of the cell lines was reversed, with SV101 and 3T6 both internalizing approximately half as much FITC insulin as the serum-sensitive 3T3 and α2-M. The cell lines all had similar amounts of total FITC insulin binding. A partial explanation for the internalization differences could be made based on the specific binding measurements, since SV101 and 3T6 had approximately two-thirds as many specific sites as 3T3. However, Aγ4 also had this low number of sites. Thus, during selection from SV101, Aγ4 did not increase its number of insulin binding sites, but did increase the amount of insulin endocytosis it underwent. At least part of this increase may have been due to fluid phase endocytosis, since Aγ4 internalized slightly more dextran than SV101 (Table 4-2). However, the total fluid phase internalization per unit surface area was unchanged while the corresponding values for WGA and insulin were significantly altered.

It should be pointed out that at 1 μM, only 25% of the insulin endocytosis by 3T3 is specific, i.e., inhibited by a 30 fold excess of unlabeled insulin (Murphy et al., 1982b). Since our fluorescent probe is biologically active, this is not likely to be due to the nature of the fluorescent insulin. Non-specific binding of radioactive insulin increases significantly when the concentration is increased from 0.1 nM to 1 nM (Jacobs et al., 1975). Thus, at the 1000-fold

higher concentration we have used, it is not surprising that we observed this high degree of non specific internalization.

Two lines of argument support the concept that the reduced insulin endocytosis we have measured in serum transformants is specific for insulin. First, competition experiments with the four cell lines showed that 20-39% of the insulin endocytosis was specific for 3T3 and Aγ4, but only 3-11% of the insulin endocytosis was specific in serum transformed 3T6 and SV101. Second, none of the non-specific probes (dextran, histone, or WGA) yielded a comparable result. However, interpretation of our results in terms of specific receptors must be reserved until analysis of both insulin and insulin like growth factor receptors is carried out with radioactive ligands.

Discussion

Our measurements of endocytosis for four cell lines indicates that transformation to serum or anchorage independence is not accompanied by significant changes in the amount of bulk endocytosis, either fluid phase or adsorptive. This is in agreement with the finding by Davies (Davies, 1980) that the rate of endocytosis of sucrose is similar for growing Balb/3T3 and SV40 transformed 3T3. Both serum and anchorage transformants show reduced receptor mediated endocytosis of α2-M. Pastan et al have reported that, when observed by indirect immunofluorescence, α2-M internalization was visibly decreased in cell lines transformed by Moloney murine sarcoma virus, relative to the parental cell lines; this was not the case for SV40 transformed Balb/3T3 or a variety of other transformants (Pastan et al., 1977). The discrepancy between their observations and our results for SV40 transformed Swiss 3T3 is probably due to the insensitivity of qualitative interpretations of microscopic images. It will be of interest to see whether other receptor-mediated processes are effected by transformation.

Serum transformation was accompanied by an increase in internalization of WGA, which was not paralleled by changes in WGA binding sites. Our results are in agreement with the suggestion that increased WA agglutinability of transformed cells results from increased

mobility of WGA binding sites and not from an increase in number of these sites (Burger, 1969). The contrast between the increased WA endocytosis and decreased α2-M and insulin endocytosis is probably due to the multivalency of WA, although it may be due to the fact that WA binds to a number of glycosylated surface proteins. Our results may be explained by the hypothesis that serum transformation leads to increased membrane fluidity and that increased fluidity facilitates aggregation (and internalization) of sites by the multivalent lectin. The results we have obtained for insulin endocytosis are in contrast to those obtained for WGA endocytosis. Serum transformed fibroblasts endocytosed less insulin than serum normal fibroblasts. Since insulin is likely to be mitogenic by virtue of its ability to bind to IGF-I receptors, it will be of interest to determine whether the same phenomenon is true for the specific internalization of IGF-I.

CHAPTER 5

DUAL PARAMETER FLOW CYTOMEZRY

This work was done in collaboration with Robert F. Murphy

Abstract

By employing the recently developed technique of staining ethanol fixed cells sequentially with fluorescein isothiocyanate and propidium iodide, we have simultaneously measured the amounts of cellular protein and DNA on a cell by cell basis, utilizing dual parameter flow cytofluorometry. In actively growing 3T3 cells some G1 phase cells contained significantly less protein than the average amount of protein contained in S, G2, and M phase cells. Quiescent 3T3 cells contained less protein than growing cells. Our results are consistent with the idea that a minimal amount of cellular protein must be accumulated before cells enter S phase. At very low concentrations of serum, SV40 transformed cells, unlike 3T3, appeared to enter S phase without the normal accumulation of cellular protein. The possible role of T-antigen in inducing this unbalanced growth is discussed.

Introduction

Cellular growth involves two fundamental processes; the duplication of cellular constituents such as proteins and membranes, and the precise duplication of chromosomal DNA. In bacteria, these two processes are tightly linked (Helmstetter et al., 1968). Under certain circumstances, DNA replication can proceed in the absence of protein synthesis. This aberrant growth appears to require the induction of the recA gene product (Lark and Lark, 1979). In mammalian cells, accumulation of protein occurs at a constant rate throughout the cell cycle, except during mitosis (Prescott, 1978). In contrast, chromosomal DNA replication occurs during a discrete

period of interphase. Although most investigators agree that a minimal amount of cellular protein is required before mammalian cells will enter S phase, it does not appear as though the accumulation of protein is a sufficient signal to the cell to enter S phase. The nature of the event leading to entry into S phase is unknown.

SV40 T-antigen can induce quiescent cells to proliferate when directly microinjected into cells. This ability, along with the well known role of T-antigen in SV40 viral DNA replication and its ability to bind to the SV40 origin or replication, has led investigators to propose that T-antigen induces DNA synthesis directly by binding to cellular origins of replication (Tooze, 1980). However, there is always a long lag period (12 hours) after the introduction of T-antigen before any DNA synthesis occurs (Tooze, 1980). Nevertheless, considerable support for the direct induction hypothesis has come from flow cytometry studies of SV40 transformed cells grown under stringent culture conditions, such as very low concentrations of serum., or isoleucine deprivation. Under such conditions, normal cells will block in G1. SV40 transformed cells will continue to synthesize DNA, and a rapid loss of viability results (Bartholomew et al., 1976; Dubrow et al., 1978).

We present evidence that SV40 transformed cells can enter S phase without the usual minimal accumulation of cellular protein. Such aberrant growth would eventually lead to cell death.

Materials and Methods

Mouse fibroblasts grown under various conditions were trypsinized with 0.025% trypsin in 0.5 mM EDTA/PBS. Care was taken to break up clumps as much as possible with gentle pipeting. The cells were centrifuged and resuspended in 30 μl of 13 mM EDTA/PBS. Then, 70 μl of ice-cold absolute ethanol was added. After one minute on ice, 100 μl of a freshly prepared FITC solution was added. RNase was added next. After 2 minutes, 100 μl of a propidium iodide solution was added. The final concentrations of the reagents were 0.33 μg/ml FITC, 3.3 μg/ml RNase, and 1.5 μg/ml propidium iodide. Then, the cells were ready for analysis by flow

cytometry. Excitation was at 488 nm as described in Chapter 4. The fluorescent beam emitted by passing cells was split with a beam splitter into two separate photomultipliers, one screened with a 540 short pass filter to detect fluorescein, and the other screened with a 620 long pass filter to detect propidium iodide.

Results

Simultaneous measurement of protein and DNA or protein and RNA has been used to analyze the balanced or unbalanced growth of mammalian cells under a variety of conditions (Darzynkiewicz et al., 1979). Although we have not done extensive controls, our results are similar to those obtained by Darzynkiewicz (Darzynkiewicz et al., 1979), and a tentative assignment of cell cycle position is shown in Figure 5-1. Figure 5-1 is a density map, i.e. darker areas represent more cells. Approximately 10,000 3T3 cells were analyzed. It is clear from this result and from the extensive results of Darzynkiewicz (Darzynkiewicz et al., 1979) that 3T3 cells accumulate a certain amount of cellular protein before entering S phase.

3T3 cells cultured in the absence of serum had less average cellular protein than 3T3 cells grown in 10% FCS, concomitant with an increase in the percentage of cells with a G2 content of DNA (Figure 5-2). In another experiment, serum-starved 3T3 cells were pulsed with serum or growth factors and then analyzed 24 hours later. As seen in Figure 5-3, control cells grown in 10% FCS showed a typical density map, whereas serum starved cells were found to be mainly in G1, with a high percentage having low amounts of cellular protein. Serum pulsed or EGF plus insulin pulsed cells had entered S phase with a concomitant increase in the average amount of cellular protein (Figure 5-3).

In a separate experiment, SV101 and 3T3 cells were serum starved for 48 hours and analyzed. DNA histograms are shown in Figure 5-4. While 3T3 cells showed an increase in the percentage of Gl cells, SV101 cells did not, even though their growth rate was lower in 0.2% FCS than in 10% FCS. This result is consistent with the result others have found, that SV40 transformed cells refuse to arrest in Gl (Bartholomew et al., 1976; Dubrow et al., 1979).

When cellular protein was simultaneously analyzed, SV101 cells displayed a significant decrease in the average amount of cellular protein, similar to 3T3 cells (data not shown). However, unlike 3T3 cells, SV101 was capable of entering S phase without an accumulation of the standard amount of cellular protein (Figure 5-5). This unbalanced growth has been observed in other systems, e.g. adenovirus 2 infected cells (Pochron et al., 1980). We have repeated this experiment several times for SV101 cells, and our experience has been that very low (0.2% or 0.1%) serum concentrations are required to see this effect.

Aγ4 also did not show an increase in the percentage of Gl cells upon serum starvation, even though there was a net loss in cell number (Figure 5-6). Aγ4 shows unbalanced growth even in 10% FCS. It should be noted that Aγ4 cultures often show a lot of floating cells. In Figure 5-7, Aγ4 cells in 10% FCS were simultaneously analyzed for cell size and cellular protein. Two populations were observed, one corresponding to a low protein/size ratio. Actually, light scattering is affected by other parameters besides cell size, such as the nuclear/cytoplasmic ratio. The meaning of this result is therefore not clear at the present time.

Discussion

T-antigen positive cells showed unbalanced growth, unlike 3T3 cells, which can block in G1 with a concomitant reduction in the amount of cellular protein. The ability of T-antigen positive cells to enter S phase without accumulation of cellular protein is similar to the ability of bacteria to initiate chromosomal DNA synthesis in the absence of protein synthesis if the recA gene product is induced (Lark and Lark, 1979).

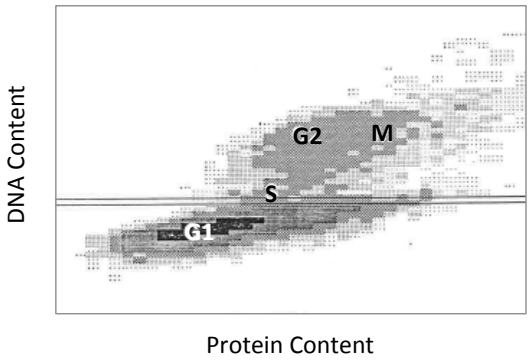

Figure 5-1. Actively growing Swiss 3T3 cells were fixed in 70% ethanol and stained sequentially with fluorescein isothiocyanate (which reacts with amino groups) and propidium iodide (which intercalates into DNA). The emission spectra of these two fluorescent molecules are sufficiently different so that there is only a minimal amount of spillover (Note the slight slant of the density map). The putative cell cycle appointments were made only because of the extensive work done by Darzynkiewicz et al. (Darzynkiewicz et al., 1979) and more recently by Crissman et al. (Crissman et al., 1981).

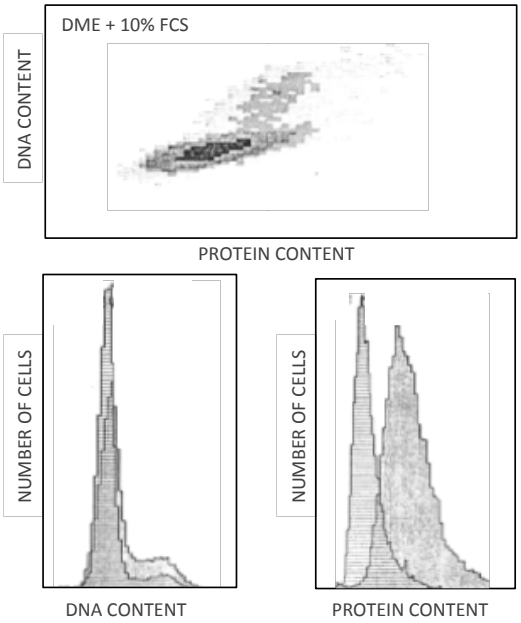

Figure 5-2. In the top panel, a graph similar to the one in Figure 5-1 is presented. On the bottom are comparisons of the DNA and protein content profiles of actively growing 3T3 (darker shading) with the DNA profile of serum-starved 3T3 (horizontal lines). The serum-starved cells have a greater percentage of Gl phase cells than does the actively growing population. Concomitant with this block in G1, the serum starved cells have less cellular protein than actively growing cells.

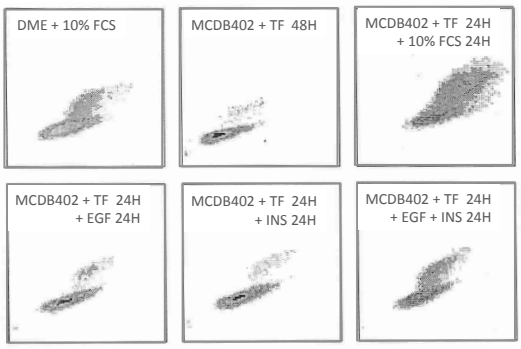

Figure 5-3. DNA (y-axis) and protein content (x-axis) profiles. The top left hand panel is control 3T3 cells kept in 10% FCS/DME. The panel following it on the right represents cells that were starved for 48 hours with no serum (MCDB 402 plus transferrin). After starving for only 24 hours, the cells were then pulsed with serum, and by 24 hours a good many of them were found in S, G2 and/or M. Insulin and EGF could only partially substitute for serum.

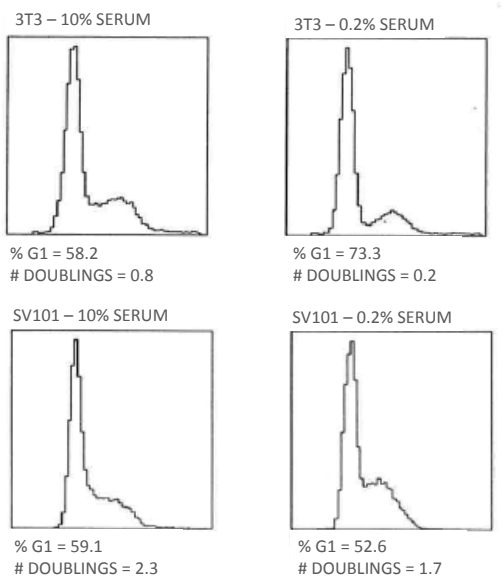

Figure 5-4. The propidium iodide emission was analyzed separately for Swiss 3T3 and SV101, and standard DNA profiles were determined. Note that 3T3 increases the percentage of cells in G1 in response to serum deprivation, whereas SV101 does not, even though its growth rate is slower in 0.2% serum.

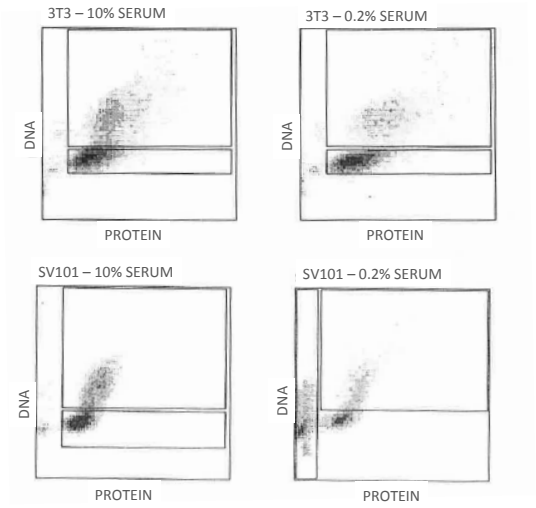

Figure 5-5. From the same experiment analyzed in Figure 5-4, except that both propidium and fluorescein emissions were measured and tabulated simultaneously. Note that SV101 contains a second peak of cells with a less than average amount of protein, and that some of these cells have more than a G1 content of DNA.

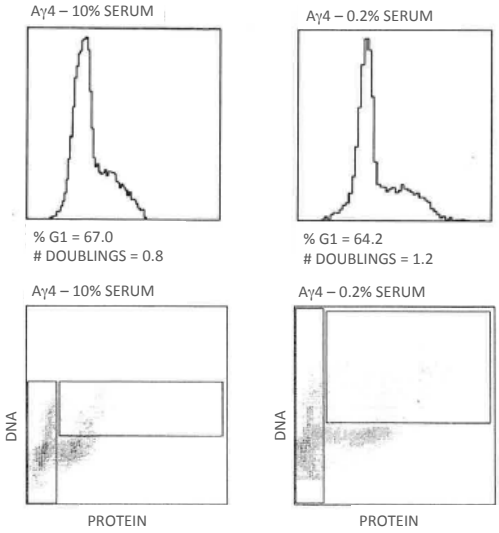

Figure 5-6. Aγ4 cells were analyzed in the same experiment with 3T3 and SV101 that was shown in the previous Figures. Even though Aγ4 was dying in low serum, it did not increase the percentage of cells in G1. Aγ4 shows the second population of cells even in 10% FCS. This was not due to dead cells or poor handling, which invariably reveals itself in the light scattering profile.

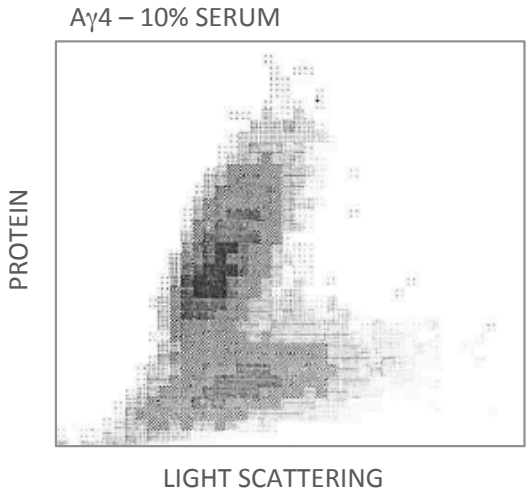

Figure 5-7. Aγ4 cells show two peaks that have the same amount of light scattering, but different amounts of protein..

CHAPTER 6

TEMPERATURE SENSITIVE LOSS OF THE PDGF REQUIREMENT IN tsA SV40 TRANSFORMED 3T3 CELLS

This work was done in collaboration with Suzie Chen

Abstract

3T3 cells transformed by the temperature sensitive SV40 mutants tsA209 and tsA58 displayed a temperature sensitive loss of density-dependent growth inhibition. The tsA209 and tsA58 transformants expressed T-antigen at the non-permissive temperature, indicating that the temperature sensitive phenotype resulted from the inactivation of T-antigen rather than the loss of expression. We examined the temperature sensitivity of the PDGF requirement in these cells. 3T3 cells require platelet derived growth factor for optimal growth in serum free medium supplemented with transferrin, EGF, and insulin. The PDGF requirement for 3T3 cells was found to be more stringent at higher cell densities, extending our previous observations that saturation density and the PDGF requirement were linked in a set of revertants derived from SV40 transformed 3T3 (Chapter 3). The wild type SV40 transformed 3T3 cell line displayed a significantly reduced PDGF requirement at both the non-permissive and permissive temperatures, in contrast to the temperature sensitive transformants that displayed a temperature sensitive loss of the PDGF requirement.

Introduction

Early studies with temperature sensitive mutants of polyoma virus demonstrated that polyoma large T-antigen was required to transform fibroblasts (Fried, 1965). These same studies indicated that large T-antigen was not required once the cell was stably transformed. It

now appears likely that large T-antigen plays a role in the integration of polyoma DNA into the host genome, but that the maintenance of the transformed phenotype is carried out by the polyoma middle T-antigen (Carmichael et al., 1982). In contrast, studies with the temperature sensitive mutants of SV40 virus that effect SV40 large antigen have shown that T-antigen plays a role in both the initiation and the maintenance of the transformed phenotype (Tooze, 1980). There have been some reports of temperature insensitive transformants induced by tsA SV40, but these temperature insensitive transformants may result from SV40 gene amplification (Hiscott et al., 1980). In the one case studied in detail, the one temperature insensitive line isolated out of sixteen transformants displayed an unusually high level of T-antigen expression at the non-permissive temperature (Brockman, 1978). This apparent gene dosage effect may underlie the ability of super T-antigens to act as more potent inducers than T-antigen of the transformed phenotype (Chen et al., 1981; Clayton et al., 1982), although other explanations are likely as well.

In tsA transformants, one property found to be temperature sensitive is the ability of the cells to grow to high saturation densities. It has been suggested that part of the ability of transformed cells to grow to high saturation densities is their insensitivity to cell shape regulation, which has been shown to directly affect the growth of both fibroblasts and endothelial cells (Folkman and Moscona, 1978). However, there is a wide body of evidence suggesting that density dependent growth inhibition is due at least in part to the depletion of serum growth factors, and that transformed cells grow to high saturation densities because of the loss of the requirement for particular growth factors (Holley, 1975). Serum growth factors can cause normal cells to grow to much higher saturation densities, and normal cells but not transformed cells can deplete serum of growth factors needed for growth of cells at low densities (Holley and Kiernan, 1968). Recently, Vogel et al. (1980) demonstrated that the final saturation density of Swiss 3T3 fibroblasts was determined by the amount of PDGF in the medium. In addition, it appears as though SV40 transformed cells secrete a PDGF-like

molecule into the culture medium, concomitant with a 90% reduction in measurable PDGF receptors (A. Vogel, personal communication).

Materials and Methods

The tsA209 transformed derivative of Swiss 3T3 (A209A) was obtained in this laboratory by the isolation of a dense focus following infection of 3T3 cells with at 33° C, and its subsequent recloning in soft agarose at 33° C. The A58D cell line was obtained by isolation of a dense focus following infection of 3T3 at 33° C with tsA58 SV40, and subsequent recloning on plastic at 33° C. SV101 is a standard SV40 3T3 transformant (Todaro et al., 1964). The non-permissive temperature of 39° C was employed since in our hands mouse cells are less viable at higher temperatures. The techniques for growing cells in serum free medium are fully described in the Materials and Methods section of Chapter 1. Immunoprecipitations were kindly done by Suzie Chen, essentially as described (Chen et al., 1981).

Results

As seen in Table 6-1, the saturation density of the two temperature sensitive transformants is significantly more temperature sensitive than the saturation densities of either 3T3 or SV101. The slight temperature sensitivity of both 3T3 and wild type SV40 transformed 3T3 has been noted by other investigators (Brockman, 1978). The A209A cell line does not grow to very high saturation densities at 33° C, unlike A58D or SV101.

Both A58D and A209A continue to express T-antigen at the non-permissive temperature of 39° C, even after several days (Figures 6-1 and 6-2). It is important to distinguish between continued expression of T-antigen and the inactivation of T-antigen in tsA transformants, as both types of phenomena have been found in tsA transformants (Tooze, 1980).

In the first experiment designed to test the temperature sensitivity of the PDGF requirement, the cells were plated at a density of 10^4/35 mm dish. This density was too low to observe a maximal differential response to PDGF, as will be discussed later. Nevertheless, both A209A

Table 6-1. Saturation Density is Temperature Sensitive in SV40 tsA Transformants

Cell Line	Saturation Density		
	33° C	39° C	Ratio (39° C/33° C)
Swiss 3T3	4.1×10^4	5.0×10^4	0.82
SV101	4.2×10^5	3.2×10^5	0.76
A58D	3.2×10^5	9.7×10^4	0.30
A209A	1.2×10^5	5.4×10^4	0.45

Cells were plated at a density of 10^5 / 35 mm dish in 10% FCS/DME and fed twice weekly. Counts were made on days 4, 7, and 9 for cells growth at 39° C and on days 4,8, and 11 for cells growth at 33° C. The final saturation density was reached (within 10% variation) by day 8, with the exception of SV101 cells grown at 33° C, which peeled off the dish after refeeding on day 5. As a result, the saturation density reported represents the day 5 count.

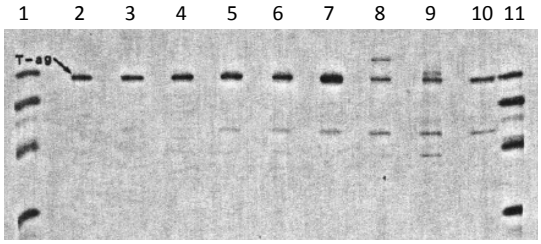

Figure 6-1. Immunoprecipitation detection of SV40 T-antigen in A209A. Lane 1 = markers, Lane 2 = A209A incubated at 33° C, lanes 3 through 7 are A209A after shift to 39° C for one day (Lane 3), two days (Lane 4) , three days (Lane 5), five days (Lane 6), and eight days (Lane 7). Lanes 8 through 9 are other cell lines. A209A may actually have higher levels of T-antigen after incubation at 39° C.

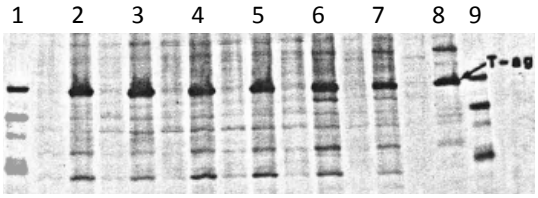

Figure 6-2. Immunoprecipitation detection of SV40 T-antigen in A58D. Lanes 1 and 9 are markers. Lane 2 = A58D incubated at 33° C, lanes 3 through 8 are A58D after shift to 39° C. Lane 3, one day; lane 4, two days; lane 5, four days; lane 6, six days; lane 7, eight days; lane 8, nine days.

and A58D displayed a higher degree of temperature sensitivity than either 3T3 or SV101 (Table 6-2).

That the PDGF requirement is sensitive to inoculation density is shown in Table 6-3. Note that the ability of the defined medium to support the growth of 3T3 drops off at lower inoculation densities, even in the presence of PDGF. Also, as shown in Chapter 1, the clonal growth of 3T3 in the defined medium is hampered, whereas it grows rapidly at higher densities. The meaning of this difference is not clear. Nevertheless, this experiment showed that the

Table 6-2. Temperature Sensitivity of the PDGF Requirement I

Cell Line	Doublings						
	+PDGF		-PDGF		Relative Growth Rate (-PDGF/+PDGF)		
	33° C	39° C	33° C	39° C	33° C	39° C	Ratio (39° C/33° C)
Swiss 3T3	3.6	2.5	2.3	1.5	0.6	0.6	0.94
SVI01	7.1	6.9	7.1	6.3	1.0	0.9	0.91
A58D	4.9	4.3	4.5	3.3	0.9	0.8	0.84
A209A	3.7	4.0	3.3	2.0	0.9	0.5	0.39

The following protocol was carried out in parallel at both 33oC and 39oC. Cells were plated at a density of 1 x 10^4 / 35 mm dish in serum free MCDB 402 supplemented with 10 μg/ml insulin, 40 ng/ml EGF, and 5 μg/ml transferrin. Dishes were coated sequentially with 0.1% poly D lysine and 1 μg/cm^2 fibronectin prior to plating the cells. Cell counts were made 24 hours later, at which time 60 ng/ml partially purified PDGF was added to half of the dishes. 6 days later, duplicate plates for each condition were scored. The numbers were converted into doublings, and the mean values obtained are reported in this table.

Table 6-3. Inoculation Density Effects the PDGF Requirement of 3T3 Cells

Inoculation Density (cells / 60 mm dish)	Doublings		Relative Growth Rate
	+PDGF	-PDGF	
3 X 10^4	3.6	1.4	0.39
1 X 10^4	3.3	1.7	0.52
3 X 10^3	2.0	1.1	0.55

3T3 cells were plated at various densities onto poly D lysine/fibronectin coated dishes under the conditions described in the legend to Table 6 2, except that the cells were grown at 37° C. Growth was monitored in a 4 day assay.

differential growth response to PDGF was accentuated at higher densities. At 5 x 10^4 cells / 35 man dish, the PDGF requirement for 3T3 was very strong, about 0.1 relative growth rate (Chapter 1).

Hence, I repeated the experiment, this time plating the cells at 5 x 10^4 cells / 35 mm dish. The temperature sensitivity of the PDGF requirement was more dramatic in the tsA transformants under these conditions (Table 6-4). Unfortunately, 3T3 also showed temperature sensitivity, somewhat detracting from the temperature-insensitivity of SV101 (Table 6-4). It should be noted that 3T3 grows to higher densities at 33° C than it does at 39° C (Table 6-1).

The clear difference between both temperature sensitive transformants and SV101 argues that a functional T-antigen is required for the loss of the PDGF requirement.

Table 6-4. Temperature Sensitivity of the PDGF Requirement II

Cell Line	Doublings				Relative Growth Rate (-PDGF/+PDGF)		
	+PDGF		-PDGF				
	33° C	39° C	33° C	39° C	33° C	39° C	Ratio (39° C/33° C)
Swiss 3T3	2.7	2.3	1.2	0.4	0.44	0.17	0.39
SVI01	4.0	3.1	3.7	3.0	0.92	0.97	1.05
A58D	2.9	2.4	2.3	1.0	0.79	0.42	0.53
A209A	2.5	1.3	1.2	0.1	0.48	0.08	0.17

Conditions were the same as described in the legend to Table 6-2, except that growth was monitored for only 4 days and the inoculation density was elevated to 5 x 10^4 cells / 35 dish.

In contrast to the complete restoration of a normal PDGF requirement observed for A209A at 39° C, A209A as well as A58D remained relatively temperature insensitive in regard to the insulin requirement (Table 6-5). However, the not insignificant temperature-sensitivity of A209A's insulin requirement would also argue that T-antigen plays at least some role in lowering the insulin requirement in SV40 transformed cells.

Table 6-5. Temperature Sensitivity of the Insulin Requirement

Cell Line	Doublings				Relative Growth Rate (-Insulin/+Insulin)		
	+Insulin		-Insulin				
	33° C	39° C	33° C	39° C	33° C	39° C	Ratio (39° C/33° C)
Swiss 3T3	2.7	2.3	1.2	0.4	0.44	0.17	0.39
SVI01	4.0	3.1	3.7	3.0	0.92	0.97	1.05
A58D	2.9	2.4	2.3	1.0	0.79	0.42	0.53
A209A	2.5	1.3	1.2	0.1	0.48	0.08	0.17

Conditions were identifical to those described in the legend to Table 6-2, except that growth was monitored for only 4 days and the inoculation density was elevated to 5 x 10^4 cells / 35 dish.

It should be noted that different temperatures inhibit different functions of SV40 T-antigen. Whereas viral DNA synthesis is completely inhibited at 39.5 ^{o} C, the induction of host cell DNA synthesis is not. At 41^{o} C, the induction of host cell DNA synthesis becomes inhibited (Tooze, 1980). This high temperature is lethal for mouse cells, preventing us from further exploring the temperature sensitivity of the insulin requirement in tsA mouse transformants.

Discussion

Our results indicate that the reduced PDGF requirement of SV40 transformed cells is mediated by T-antigen. The ability of T-antigen to enhance the transcription of cellular genes (Schutzbank et al., 1982) may underlie this phenomena. Two cellular genes that might be effected in this manner are the gene that has been implicated in mediating the mitogenic response to PDGF (Stiles and Smith, 1981), and the gene that codes for an endogenous PDGF like molecule. Vogel and Ross have found that SV40 transformed fibroblasts display low amounts of PDGF binding, concomitant with their secretion of a molecule immunologically related to PDGF (A. Vogel, personal communication).

In my experiments, unconcentrated conditioned medium from SV101 cells did not substitute for PDGF for the growth of 3T3 cells, but did inhibit the response of 3T3 cells to PDGF (Table 6-6). SV101 cells secrete copious amounts of plasminogen activator (Rifkin and Pollack, 1977), and this protease may inactivate PDGF. Concentrated conditioned medium from SV101 cells did partially substitute for PDGF (Figure 6-3). We are presently involved in a collaboration with Vogel and Ross to determine whether our temperature sensitive cell lines are temperature sensitive for the secretion of a PDGF like molecule.

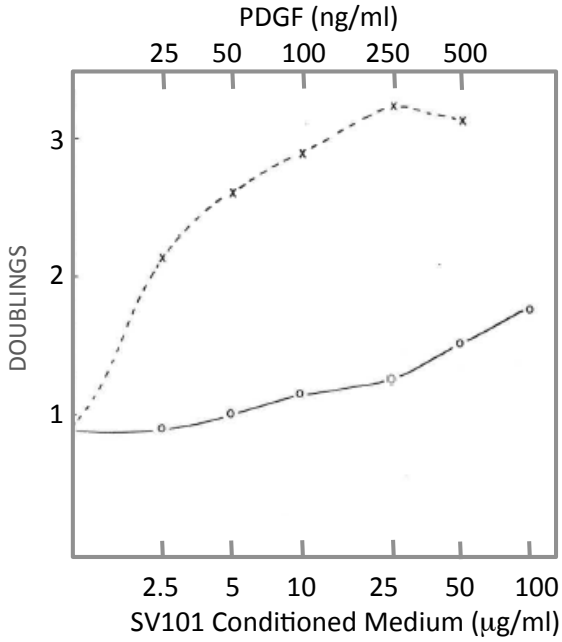

Figure 6-3. Swiss 3T3 cells were plated out at high density as described in the legend to Table 6-6. On day 1, the indicated amount of either PDGF (top scale) or concentrated SV101 conditioned medium (bottom scale) was added. 3 days after adding the factors, cell counts were performed. To collect conditioned medium, MCDB402 with freshly dissolved ferrous sulfate was used without any supplementation. SV101 conditioned medium was collected continuously once every 48 hours for 3 weeks with the same cultures (2 x 10^7 cells/ 100 mm dish) .

Table 6-6. Conditioned Media Experiment

Conditioned Medium	Doublings	
	-PDGF	+PDGF
Control	0.1	1.6
NIH 3T3	0.5	1.8
SVIns4	0.4	1.7
SV101	0.1	1.0

The growth response of 3T3 to PDGF was measured in a 76 hour assay as previously described, with an inoculation density of 5 x 10^4 cells / 35 mm dish. Control plates received untreated MCDB 402. The other plates received MCDB 402 that had been conditioned by incubation for 48 hours with 8 x 10^6 cells (indicated in the table above) per 150 mm fibronectin coated dish. No growth factors wereadded during the conditioning process. Approximately 20 ml conditioned medium was recovered from each dish, centrifuged, and filtered before use. All plates were supplemented with 5 μg/ml transferrin, 40 ng/ml EGF, and 10 μg/ml insulin. SVIns4 retains a partial PDGF requirement, as discussed in Chapter 7.

CHAPTER 7

SV40 TRANSFORMATION IN SERUM FREE MEDIUM: DIRECT SELECTION FOR INSULIN INDEPENDENT TRANSFORMANTS

The following work was done in collaboration with Suzie Chen

Abstract

Infection of normal Swiss 3T3 cells with either wild type or dl884 SV40 virus induced actively growing colonies in serum free medium supplemented with transferrin, epidermal growth factor, and fibroblast growth factor. Uninfected 3T3 cells formed colonies only if insulin was added in addition to the other growth factors. This serum free assay for isolating SV40 transformants yielded T-antigen positive cell lines that were less transformed than standard SV40 transformants isolated as dense foci or as colonies in semi solid medium. It appears as though some of the properties associated with SV40 transformation, i.e. growth in semi solid medium or growth to very high saturation densities, are not obligatory responses to transformation by SV40, in agreement with earlier work from this laboratory (Risser and Pollack, 1974).

Introduction

Transformation of normal cells by tumor viruses is usually a rare event; generally only a few cells are converted to stable transformants. Extremely high doses of SV40 virions can transform approximately 50% of 3T3 cells (Todaro and Green, 1966), and high doses of Rous sarcoma virus can transform close to 100% of chick embryo fibroblasts (Varmus et al., 1984). However, when low amounts of virus are added, or when DNA is used, the transformation frequency drops (Tooze, 1980). To detect transformants at such low frequencies, it is necessary to construct a selective assay in which some form of overgrowth can be detected against a

background of normal growth inhibition. The most commonly employed assay is the dense focus assay, in which transformed colonies can be detected either by eye or microscopically against a background monolayer of density inhibited cells. The anchorage assay offers very high resolving power; i.e. 3 x 10^5 normal cells can be plated onto a 60 mm dish and remain as single cells or doublets for 3 weeks or more (Tooze, 1980).

Little attention has been paid to some of the consequences that arise when such selective assays are employed. Normally, whatever properties the transformed clone exhibits are ascribed to the presence of the viral transforming gene. This assumption is invalid in the case of SV40, since Risser and Pollack (Risser and Pollack, 1974) clearly demonstrated that SV40 transformation of both rat fibroblasts and mouse 3T3 fibroblasts did not necessarily result in their ability to grow in semi solid medium, a property invariably associated with cells transformed in the standard density assay (Tooze, 1980). However, all SV40 transformants displayed a reduced requirement for serum, indicating that this change in phenotype might be an obligatory response to SV40 transformation.

Our previous work with serum free medium indicated that the loss of a normal serum requirement and the loss of an insulin requirement for growth in defined medium were related (Chapter 3). We therefore began work on developing this observation into a selective assay for SV40 transformants.

Materials and Methods

The insulin transformants, SVInsl through SVIns7, were isolated following SV40 infection of 3T3-R, a flat, somewhat adipogenic subclone of Swiss 3T3. dl884 virus was from Bob Martin originally. The wild type virus used was the wt830 strain, the parent of dl884 (Tooze, 1980). The insulin transformants were isolated in the partially defined medium that involved serum coating dishes and the use of gimmel factor. 3T3-R was plated at subconfluent densities and infected with 50-500 pfu/ml virus 24 48 hours later. After 2 hours incubation with virus, the infected plates were washed twice with serum free DME, and then refed 10%

FCS/DME for 24 hours. At this time, control plates and infected plates were subcultured by one rinse with 0.5 mM EDTA/PBS and then allowed to detach in PBS. Cells were plated at densities ranging from 10^3 to 3 x 10^4 per 60 mm dish. All plates received MCBD 402 supplemented with 5 μg/ml transferrin, 5 μg/ml gimmel factor, 10 ng/ml FGF, and with or without 10 μg/ml insulin. 10 to 21 days later, transformed colonies growing without insulin could be detected. When these colonies reached a sufficiently large size, they were isolated with steel cloning cylinders and subsequently recloned in 10% FCS/DME.

Characterization of the Insulin Transformants

The insulin transformants were tested for the stability of their phenotype of not requiring insulin in the totally defined medium described in Chapter 1. At the time of the characterization, FGF was not available from the commercial supplier. Instead, I used 10 ng/ml vasopressin, a component of defined medium for rat fibroblasts (McClure et al., 1982). The PDGF requirement was assayed as described in Chapter 6. 5 x 10^4 cells were plated onto 35 mm dishes containing 10 μg/ml insulin, 5 μg/ml transferrin, and 40 ng/ml EGF. 24 hours later, duplicate counts were made, and half of the remaining plates received 60 ng/ml PDGF. 3 days later, counts were performed and the doublings with and without PDGF were used to determine the relative growth rate.

The insulin requirement was determined similarly, but only 5 x 10^3 cells were plated onto 35 mm dishes. The medium included 5 μg/ml transferrin, 40 ng/ml EGF, and 10 ng/ml vasopressin. 36 hours later, duplicate plates were counted and 10 μg/ml insulin was added to half of the remaining plates. 4 days later, counts were performed and the doublings with and without insulin were used to determine the relative growth rate. The saturation density measurements involved plating 10^5 cells/35 mm dish in 10% FCS/DME. Duplicate counts were made on day 4, day 7, and day 9.

As seen in Figure 5-1, the clonal growth of Swiss 3T3 cells is clearly dependent upon the presence of insulin. In contrast, SV40-infected 3T3 will form colonies in the absence of

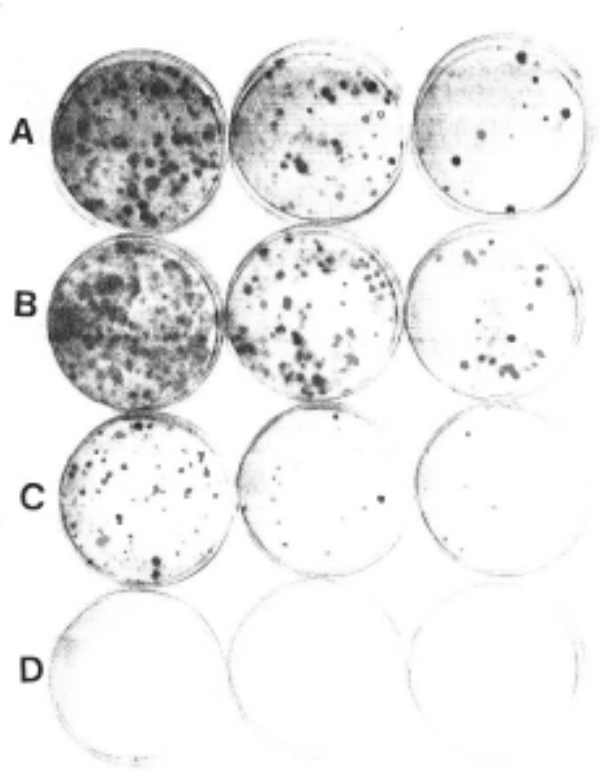

Figure 7-1. Insulin transformation assay. SV40 infected (500 pfu/cell) 3T3 or mock infected 3T3 were plated at either 30,000 cells / 60 mm dish (left hand column), 104 cells (middle column), or 3000 cells (right hand column). The bottom two rows, C and D, were not fed insulin, whereas the top two rows were fed insulin. Rows A and C were SV40 infected, rows B and D were mock infected. Note the complete absence of colony formation in mock infected 3T3 without insulin. These cultures were grown for 12 days post plating before fixation. Arno Scheller kindly provided me with some purified SV40 virus for this experiment. The medium consisted of poly D¬lysine/fibronectin coated dishes with 4 ml MCDB 402, 5 ug/ml transferrin, 40 ng/ml EGF, 10 ng/ml vasopressin, and with or without insulin as indicated.

insulin. Abortive transformation was noted in the first few days of this experiment. After 5 days, the abortively transformed colonies (which were microscopic in size) stopped growing. The visible colonies were actively growing at the time the experiment was terminated. This selective assay had enough resolving power to detect insulin transformants at least up to 1 in 10^5. Although Figure 5-1 does not reveal the appearance of somewhat denser colonies that had

grown in the SV40 infected plates with insulin at the highest inoculation density (3 x 10^4 / 60 mm dish), considerably packed colonies were present. However, some subjective judgment must be made. In contrast, there is little doubt as to the selective basis for isolating insulin transformants, given the very low background of normal 3T3 growth when insulin is left out of the medium.

Several insulin transformants have been isolated and characterized. Not all of them grow well in semi solid medium (Table 7-1).

Table 7-1. Characterization of Insulin Transformants

	Relative Growth Rate		Saturation Density	Colonies in Methocel
Cell Line	(Ins/+Ins)	(PDGF/+PDGF)		
Swiss 3T3 R	0.17	0.11	4	< .0005
SVInsl	0.45	0.51	15	0.002
SVIns2	0.37	0.47	10	0.0005
SVIns4	0.49	0.59	44	0.0005
SVIns5	0.72	0.99	90	2
SVIns6	0.54	0.72	33	20
SVIns7	0.63	0.87	62	1
SVS	0.66	0.76	81	1
SVA	0.75	0.95	87	2

*this value applies only to early passage SVIns4. Later passage SVIns4 grew well in methocel (1% efficiency). In contrast, the anchorage-dependent phenotypes of SVIns1 and SVIns2 were stable upon passage. Anchorage-dependence was determined by plating 10^5, 10^4, and 10^3 cells in methocel on 60 mm dishes that were coated with 2 ml 0.5% agarose. The plates were fed weekly with 5 ml methocel medium. Saturation densities represent 1 x 10^{-4} cells/cm^2. The growth factor requirements were determined as described in Materials and Methods.

Two of these transformants, SVInsl and SVIns2, were isolated as colonies growing without insulin after infection with the dl884 mutant SV40 virus that lacks part of the coding sequences for little T-antigen (Tooze, 1980). In particular, SVInsl and SVIns2 were the closest to the parental clone 3T3-R (a subclone of Swiss 3T3) in terms of their saturation density, anchorage requirement, and PDGF requirement. We did not isolate enough clones to make any

statistically significant statement about whether wild type SV40 insulin transformants were invariably more transformed than dl884 insulin transformants. SVIns4 and SVIns6 were considerably more sensitive to density dependent growth inhibition than either SVA or SVS, two control wild type transformants of 3T3-R isolated as a colony growing in methocel and as a colony growing as a dense focus, respectively. All of these insulin transformants were analyzed for the expression of T-antigen. All 7 insulin transformants expressed T-antigen, and in addition some of them expressed off sized T-antigens (Figures 7-2 and 7-3). On the average, the insulin transformants are not as transformed as standard transformants like SV101, SVA, or SVS. In some ways their behavior is like that of the "intermediate" transformants detected by Risser and Pollack (Risser and Pollack, 1974).

Discussion

A selective assay of high resolving power was developed for the isolation of insulin transformants. This assay may be useful in selecting transformants that do not exhibit properties that may arise as a consequence of selecting dense foci.

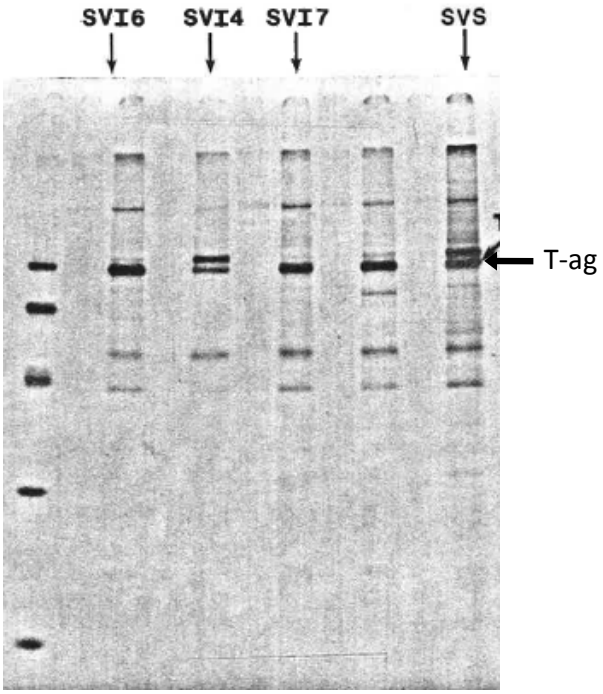

Figure 7-2. Immunoprecipitation analysis of SV40 T-antigen expression in the insulin transformants was carried out essentially as described by Chen et al. (1981).

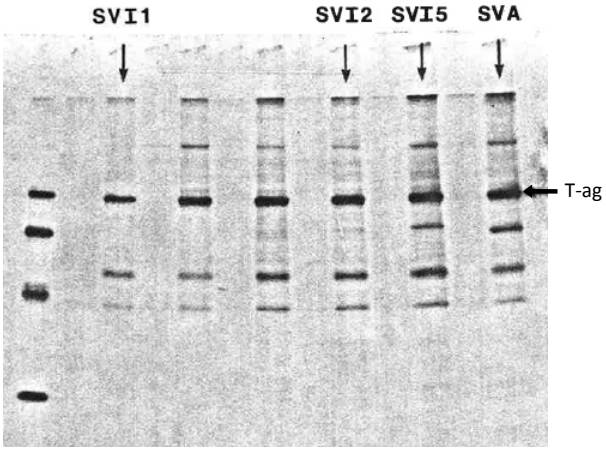

Figure 7-3. Immunoprecipitation analysis of SV40 T-antigen expression in the insulin transformants was carried out essentially as described by Chen et al. (1981).

References

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