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# HIT cells secrete β-cell mitogenic factors

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**We studied the growth characteristics of the insulin-producing HIT cells. Although successful in many cell lines such as βTC1, growth arrest could not be obtained with HIT cells left for 3 days without serum. Cytofluorometric analysis showed that about 24% of the cells continuously exposed to serum peaked in the S phase. A similar proportion was found for cells cultured for 1 or 2 days in serum-free medium. A treatment with suramin, disrupting the binding of ligands from their receptors, was associated with a rapid and transient increase in c-*fos* and c-*jun* gene expression after suramin removal, in the absence of serum. In addition, HIT cells secrete mitogenic factors, different from IGF-I or IGF-II, acting on insulin-secreting βTC1 cells and on BP-A31 fibroblasts. Chromatography of the medium conditioned by the HIT cells on gel filtration gave two major mitogenic fractions, of hydrodynamic characteristics 33 000 and 3000–10 000. The activity was heat stable and bound to heparin. Comparative studies of the self-regulatory HIT cells, with the βTC1 cells requiring external growth factors, should contribute significantly to our understanding of the regulation of β cell growth.**

**Keywords:** HIT cells; cytofluorometric analysis; insulin; β cell growth; mitosis

## Introduction

The understanding of the mechanisms responsible for differentiation and growth of the pancreatic β cell can provide important informations about β cell dysfunctions observed in the different types of diabetes. Demands for increased insulin production could be satisfied by adjusted regulation of the β cell mass and therefore the knowledge of the factors that control β cell growth is of acute importance (Hellerström & Swenne, 1985).

Increasing evidence indicate that the growth of mammalian cells is governed by a number of substances that include growth factors as well as neuroendocrine and paracrine mediators interacting with specific cell surface receptors. We have recently described that βTC1 cells derived from transgenic mice expressing hybrid insulin-SV40 T antigen genes (Efrat *et al.*, 1988) can be rendered quiescent by serum deprivation and resume their cell cycle associated with immediate-early gene expression following serum stimulation (Bréant *et al.*, 1990). This provided an interesting model for studying the factors involved in the regulation of β cell proliferation. As do βTC1 cells, HIT cells issued from SV40-transformed hamster islets (Santerre *et al.*, 1981), produce substantial quantities of insulin, have retained the glucose responsiveness and are widely used to study the mechanism of β cell function (Hill & Boyd, 1985; Ashcroft *et al.*, 1986; Hammonds *et al.*, 1987). It was of interest to evaluate if the different genetic events that have directed the immortalization and transformation of HIT and βTC1 cells could have generated distinct growth factor requirements for these two cell lines. We present here the results obtained with the HIT cells. Interestingly it was found that serum deprivation was ineffective to arrest HIT cell growth. Experimental evidences are presented in this paper that support an autonomous regulation of HIT cell cycle and a secretion of growth factor(s) acting on heterologous β and fibroblastic cell lines.

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## Results

### *Effect of serum starvation*

In mammalian cells in culture, including the insulin-secreting βTC1 cells, growth can be arrested by depletion of serum or growth factors in the culture medium. The growth characteristics of HIT cells was studied by submitting the cells to serum deprivation for 3 days, a period which corresponds to two generation times. This study was performed at three different cellular densities with serum concentrations varying from 0 to 0.25% (v/v) in the deprived medium (Figure 1). In all conditions tested, elevated [$^3$H]thymidine incorporation

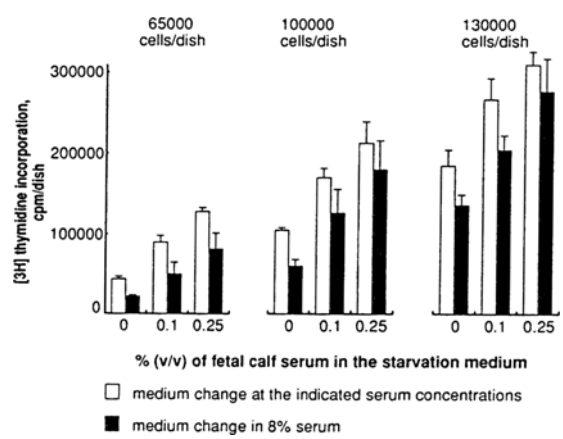


**Figure 1** Effect of serum starvation. Twenty four hours after seeding in standard medium (8% FCS), HIT cells were washed twice with serum-free medium and then incubated for 3 days at various serum concentrations (0, 0.1 or 0.25%, v/v). The medium was changed and [$^3$H]thymidine incorporation (1 μCi/ml) was allowed to proceed for the following 24 h either at the various serum concentrations (□) or in the presence of 8% FCS (■). Results are means of triplicate determinations

was achieved by the deprived cells (open symbols), indicating that, even in the absence of serum, HIT cells were still synthesizing DNA. It is noteworthy that DNA synthesis was retained even at the lowest cell density (65 000 cells/dish). Cells exhibited a higher [$^3$H]thymidine incorporation per million of cells than that expected considering cell density (compare in Figure 1 the open symbols for a given serum concentration). No further incorporation was obtained by subsequent addition of serum (black symbols) for the 24 h following the starvation period. Thymidine incorporation was even slightly below that obtained in the corresponding low serum conditions, suggesting that some substances may prevent optimal growth.

*Cell cycle analysis*

To further confirm the proliferative capacity of the HIT cells in serum-free medium, cytofluorometric analysis was performed to monitor the progression of the cells in the different phases of the cell cycle. The cell repartition was statistically not different in exponentially growing cells cultured in the presence of serum and in cells cultured for one or two days in serum-free medium (Table 1). A large proportion of cells peaked in the G1 channels (about 60%), whereas about 24% of cells were in the S phase and approximately 16% in the G2/M channels.

*Cell cycle-related gene expression*

The finding that the growth of HIT cells could not be arrested by serum starvation prompted us to investigate if the initiation of the early events of the cell cycle, i.e. the transient expression of immediate early genes, could be achieved in the absence of growth factors. The polyanionic drug suramin has among a number of biological effects, the property to reversibly dissociate ligands from their receptors and consequently to prevent the binding of the growth factors present in the culture medium (Hosang, 1985; Coffey *et al.*, 1987). HIT cells were thus treated for 24 h with suramin in the absence of serum. Whereas c-*fos* mRNA was undetectable after suramin treatment (Figure 2, lane 1), the c-*jun* gene was constitutively expressed as well as the cdc2 gene. Similar results were obtained in serum-deprived βTC1 cells (Bréant *et al.*, 1990), further documenting the constitutive expression of these genes in pancreatic β cell lines bearing the SV-40 large T antigen. As soon as 30 min after suramin removal (lane 2), a net induction of c-*fos* gene expression was observed together with an enhancement of c-*jun* mRNA accumulation. This increase of c-*fos* and c-*jun* mRNA could also be observed after stimulation of serum-free treated cells with serum for 30 min (data not shown). After 2 h in serum-free medium (lane 3), the expression of these two genes returned to basal levels which remained unchanged for 24 h (lane 4). The c-*myc* gene was poorly expressed in the suramin-treated HIT cells and mRNA levels remained very low when the drug was removed (Figure 2, lanes 1 to 4). Under these experimental conditions, cdc2 and insulin mRNA levels were unchanged, except at 24 h where insulin gene expression slightly decreased. Hybridization with the α-tubulin probe is presented as an internal control.

*Mitogenicity of the HIT conditioned medium*

The possible production of auto-stimulatory mitogens could account for the failure of HIT cells to enter quiescence in serum-free medium. We therefore collected conditioned medium from cells which had been trypsinized and replated in serum-free medium. This medium was assayed for its ability to induce [$^3$H]thymidine incorporation in two heterologous cell lines, the βTC1 cell line and the fibroblastic BP-A31 cell line, both strictly dependent on exogenously added serum or growth factors for promoting DNA synthesis. Cells were rendered quiescent by serum deprivation and then submitted to various concentrations of the HIT-conditioned medium (CM) for 24 h in the presence of [$^3$H]thymidine (Figure 3). As compared to serum-free medium, the HIT medium strongly stimulated [$^3$H]thymidine incorporation in both cell lines, indicating that the HIT cells had secreted growth factors eliciting the resumption of βTC1 and BP-A31

**Table 1** Cytofluorometric analysis of HIT cell cycle

| | *Percent of cells in each phase* | | |
|---|---|---|---|
| *Treatment* | *G1* | *S* | *G2/M* |
| 8% FCS 48 h | 59.1±2.7 | 24.3±2.4 | 15.9±0.4 |
| 0% FCS 24 h | 60.4±3.7 | 23.7±2.3 | 14.6±0.8 |
| 0% FCS 48 h | 55.5±3.7 | 25.5±1.6 | 18.7±1.2 |

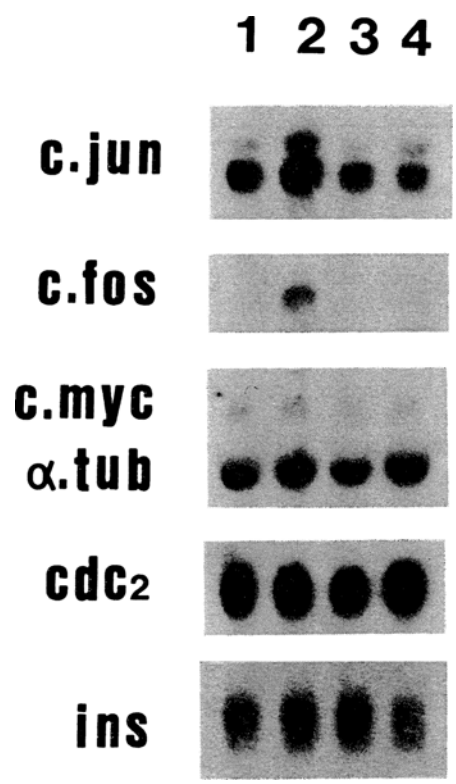


**Figure 2** Cell cycle-related gene expression. HIT cells were treated for 24 h with suramin and RNA was extracted immediately (lane 1), 30 min (lane 2), 2 h (lane 3) or 24 h (lane 4) after suramin removal. A 20 μg sample of total RNA analysed by Northern blot was hybridized with the indicated radiolabeled probes. Sizes of the mRNAs: c-*jun* 2.7 and 3.2 kb, c-*fos* 2.3 kb, c-*myc* 2.2 kb, α-tubulin 1.8 kb, cdc2 1.7 kb and insulin 0.56 kb. Exposure time was overnight except for c-*myc* and α-tubulin (2 days)

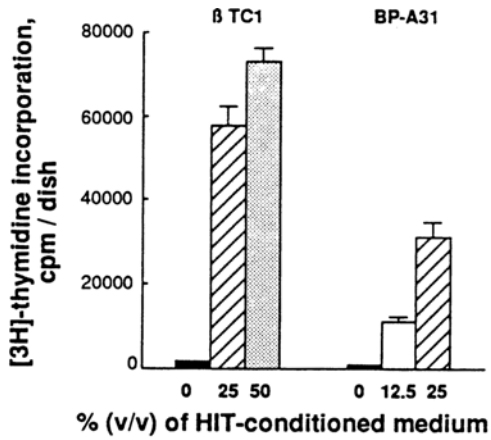


**Figure 3** HIT cells secrete growth-promoting factors. The conditioned medium from HIT cells that had been trypsinized and replated in serum-free medium was collected 24 h after seeding, centrifuged to remove any detached cells and frozen at −20°C until use. βTC1 and BP-A31 cells were rendered quiescent by serum-deprivation in their respective culture medium and submitted to the HIT-conditioned medium which was thawed at 37°C for 30 min. [$^3$H]thymidine (1 μCi/ml) was added concomitantly for the 24 h of the stimulation. ■: serum-free RPMI medium; □: 12.5% conditioned medium in RPMI; ▨: 25% conditioned medium in RPMI; ▩: 50% conditioned medium in RPMI

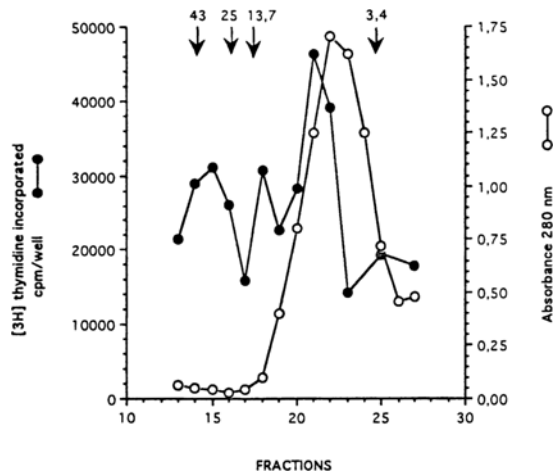


**Figure 4** Gel filtration of HIT conditioned medium. The conditioned medium after lyophylization was applied onto a Sephacryl S100 column in PBS. Collected fractions (1.5 ml) were frozen and their mitogenic activity was assayed on quiescent βTC1 cells at a 1/4 dilution in RPMI (●). Absorbance at 280 nm is shown by ○. Calibration with appropriate molecular-weight standards is indicated by arrows

cell division cycle. Micromolar concentrations of insulin can direct BP-A31 cells towards DNA synthesis through type-I IGF receptors (Buchou *et al.*, 1989). However insulin is secreted at nanomolar concentrations in the HIT medium (see Materials and methods) and is therefore not likely to mediate the mitogenic effect observed on the fibroblastic cells.

### *Gel filtration of the HIT conditioned medium*

To further characterize the factors produced by the HIT cells, the conditioned medium was subjected to gel filtration chromatography and the mitogenicity of the different fractions was assayed on quiescent βTC1 cells. Two major peaks of activity were obtained, of estimated molecular mass 33 000 and 3000–10 000 Da (Figure 4). After treatment of the conditioned medium for 20 min at 56°C or 3 min at 100°C, 85–90% of the mitogenic activity was recovered, suggesting that it was heat-stable.

### *Expression of Insulin-like growth factors in HIT and βTC1 cells*

Insulin-like Growth Factors (IGFs) have been shown both to be produced by pancreatic islets and to regulate islet growth (Hill & Hogg, 1991; Maake & Reinecke, 1993). Since the molecular mass of the putative HIT growth factor lies in the range of 3000–10 000 which is close to that of IGFs, it was of interest to measure the secretion of IGF-I and IGF-II together with the accumulation of their corresponding mRNAs. HIT cells as well as βTC1 cells poorly secreted IGF-I (0.02 to 0.08 ng/ml). In line with these results, IGF-I mRNA could not be detected in the two cell lines in all culture conditions studied (result not shown). The HIT-conditioned medium contained barely detectable levels of IGF-II (0.6 ng/ml) and IGF-II mRNA was not detectable in HIT cells, even after

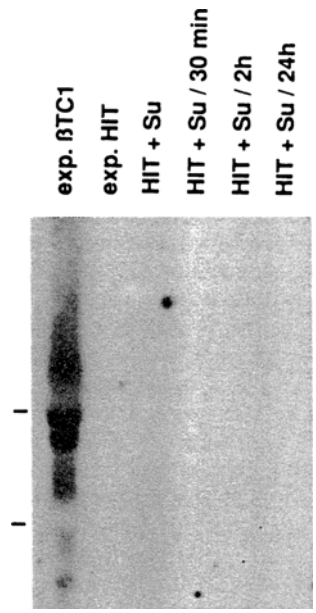


**Figure 5** Expression of IGF-I and IGF-II in HIT and βTC1 cells. A 20 μg sample of total RNA extracted from βTC1 or HIT cells cultured in the continuous presence of serum (exp. βTC1 and exp. HIT) was analysed by Northern blotting. Similarly to the experiment described in Figure 3, RNA from HIT cells treated for 24 h with suramin were extracted either immediately (HIT + Su) or 30 min, 2 h or 24 h after suramin removal. The radiolabeled insert corresponding to the human IGF-II cDNA hybridized to several mRNA species of 6 kb, 5 kb, 4.8 kb and 2.2 kb in βTC1 cells only (lane exp. βTC1). On the left is indicated the migration of 28S and 18S ribosomal RNAs

prolonged exposure of the autoradiogram (Figure 5). It should be noted that the human IGF-II cDNA probe used hybridized to two mRNA species in fetal hamster liver (result not shown). In contrast, βTC1 cells accumulated high levels of several IGF-II mRNA species when cultured in the presence of serum (Figure 5, first lane) as well as after 3 days in serum-free medium (not shown). Under these latter conditions, βTC1 cells secreted substantial amounts of IGF-II

(13–25 ng/ml). Taken together these results suggest that the mitogenic effect of the HIT conditioned medium on βTC1 cells is not likely to be mediated by IGF-I or II.

The ability of the secreted mitogens to bind to heparin was determined. The majority (100–110%) of the starting mitogenic activity was eluted with high salt concentration from the heparin-agarose column. This result suggests that the secreted factors are likely to belong to the class of the heparin-binding growth factors. Interestingly, betacellulin, a 32 000 dalton glycoprotein binding to heparin, has already been shown to be secreted by a pancreatic β cell line (Shing *et al.*, 1993). Betacellulin mRNA could not be detected in HIT cells by Northern blot analysis of polyadenylated RNA (result not shown).

## Discussion

In this report, we present data in favour of an autonomous regulation of HIT cell growth. Serum-deprivation was ineffective to arrest thymidine incorporation into DNA in these cells. This constituted a striking difference between HIT cells derived from SV40-transformed hamster islets and βTC1 cells issued of transgenic mice carrying hybrid insulin-T antigen gene constructs. In βTC1 cells, quiescence was reached after a few days in serum-free medium, indicating that these transformed cells still required serum growth factors to resume the cell cycle. It could be suggested that additional genetic events have directed the HIT cells towards a more transformed phenotype, leading to a relative serum independence. The different methods of cell cycle analysis that we have used (TCA-precipitable thymidine incorporation and cytofluorometry), gave consistent results which indicate that HIT cells are able to resume their cycle in the absence of any growth factors to the same extent as in the presence of serum. The constitutive expression of c-*jun* was also observed in βTC1 cells and likely to be due to the presence of SV-40 large T antigen, as suggested by Piette *et al.* (1988). The role of this gene in confering a serum independence in HIT cells is still unclear since βTC1 cells, carrying SV-40 large T antigen under the control of the rat insulin II promoter, have still retained their need for serum factors; however it has been shown in various cell lines that SV-40 could induce the secretion of PDGF-like substances (Bleiberg *et al.*, 1985; Werner *et al.*, 1989). The transient induction of c-*fos* and the enhancement of c-*jun* mRNA accumulation, as early as 30 min after suramin removal in serum-free medium is another argument which supports the autonomous regulation of HIT cell growth. The transient expression of these two genes is in line with that observed in other cellular systems (Treisman, 1985, 1986; Lau & Nathans, 1987; Angel *et al.*, 1988) and was also demonstrated during the resumption of βTC1 and RIN 5AH cell cycle (Bréant *et al.*, 1990; Petersen *et al.*, 1990 respectively), but was dependent upon the addition of serum. One could suggest that in the HIT cells, growth factors are released from the suramin complex after suramin removal, thus allowing free access of the autonomous mitogens to their receptors. Whereas the induction of the c-*myc* gene is an early event controlling cell cycle resumption and was shown to be induced upon glucose stimulation in RIN cells (Yamashita *et al.*, 1988), this gene was poorly expressed and did not appear to be regulated during HIT cell cycle. Similar results were previously reported in the βTC1 cell line (Bréant *et al.*, 1990). This early responsive gene is characteristic of the GO/G1 transition, but can also be activated throughout the cell cycle, provided the presence of a mitogenic stimulation (Thompson *et al.*, 1985; Bravo *et al.*, 1988).

The failure of HIT cells to enter quiescence, as well as the induction of cell cycle-related gene expression in serum-free medium, could be attributed to an autonomous regulation of HIT cell cycle. Indeed, the secretion of mitogens was demonstrated by the high efficiency of HIT-conditioned medium to direct quiescent cells towards DNA synthesis. It cannot be ruled out that insulin itself, secreted at less than the nanomolar range in the medium could be partly responsible for the stimulation of BP-A31 cell DNA synthesis. This hypothesis seems however unlikely since in these cells, effective mitogenic effect is observed with micromolar concentrations of insulin, likely to interact with type I-IGF receptors (Buchou *et al.*, 1989). Thus other factors, apart from insulin, are potential mitogenic agents and this is further sustained in the case of βTC1 cells, which secrete higher concentrations of insulin than the HIT cells, in serum-free medium (C. Lavergne, personal communication). It seems rather unlikely that the mitogenic effect of the HIT-conditioned medium could be attributed to Insulin-like Growth Factors I and II, which are secreted at a low level. The endogenous production of Insulin-like Growth Factors has been reported in human fetal pancreas and predominantly in the β cells, associated with the finding of mRNA for both IGF-I and -II, with a marked prevalence for IGF-II (Hill & Hogg, 1991). The elevated expression of IGF-II mRNA and the release of IGF-II we observed in βTC1 cells, closely fit the above findings on the pancreatic tissue. Moreover, many lines of evidence support the concept that IGF-binding proteins (IGF-BPs) modulate the mitogenic activity of IGFs, in both stimulatory and inhibitory ways (Baxter & Martin, 1989; Hogg *et al.*, 1993; Chen *et al.*, 1994). The possible production of IGF-BPs, in the medium conditioned by the HIT cells could modulate the mitogenic activity of IGF-II secreted by the βTC1 cells. This could be an attractive alternative for regulating βTC1 cell DNA synthesis.

The finding that cultured β cells secrete mitogenic factors has recently been demonstrated. Betacellulin, a 32 000 dalton glycoprotein has been purified from the conditioned medium of βTC3 cells (Shing *et al.*, 1993). These cells arise from transgenic mice carrying an insulin-promoted large T antigen gene, but from another cell lineage than βTC1 cells (Efrat *et al.*, 1988). It should be noted that the growth of βTC3 cells could not be arrested in low-serum-containing medium (Bréant *et al.*, 1992), unlike the βTC1 cell line. Betacellulin, member of the epidermal growth factor family, is a potent mitogen for vascular smooth muscle as well as retinal pigment epithelial cells but its potential effect on pancreatic β cells has not yet been demonstrated. Although betacellulin mRNA could not be detected in HIT cells, it cannot be excluded that the activity we found in the high molecular-weight range could correspond to a betacellulin-like factor. This hypothesis as

well as the full characterization of the factor(s) secreted by the HIT cells remain to be elucidated.

## Materials and methods

### *Materials*

Cell culture media were obtained from Intermed (Noisy le Grand, France). Fetal calf serum (FCS) was from Boehringer Mannheim (Meylan, France). [$^3$H]thymidine (5–20 Ci/mmol) was purchased from Amersham (Les Ulis, France). Suramin was a gift of Bayer-Pharma, Puteaux, France. Other chemicals were of reagent grade.

### *Cell culture*

HIT cells were routinely cultured in RPMI 1640 containing 25 mM glucose and βTC1 cells in DMEM. The fibroblastic cell line BP-A31, obtained by benzo [α] pyrene transformation of Balb/c 3T3 cells clone A31 (Dubrow *et al.*, 1979), was cultured in MEM-α medium. All cell lines were cultured in their respective medium supplemented with 8% Fetal Calf Serum (FCS), 100 U/ml Penicillin and 100 mg/ml Streptomycin in a humidified atmosphere of 5% CO2 in air at 37°C. They were plated in Corning 75 cm$^2$ flasks and passed once a week with trypsin/EDTA. The medium was changed every 2 to 3 days. HIT cells subclone T15 were generously provided by Prof. C.B. Wollheim (Geneva, Switzerland) and used from passage numbers 70 to 74. Insulin secretion and content were controlled periodically by radioimmunoassay using rat insulin as standard; as an indication, HIT cells secrete 3.46 ± 0.19 mUnits insulin/million cells/24 h when cultured in 8% FCS and 0.74 ± 0.05 mUnits insulin/million cells/24 h after 2 days in serum-free medium.

### *Measurement of the rate of DNA synthesis*

For studies of mitogenic effects, HIT cells were seeded in 24-well dishes (100 000 cells/well in 1 ml volume, unless otherwise stated in the figure legends) in medium supplemented with 8% FCS. Twenty-four hours after seeding, cells were washed twice with 1 ml serum-free medium and then incubated for several days as indicated in the figures. βTC1 and BP-A31 cells were rendered quiescent by deprivation of serum in their respective culture medium for 3 days, as previously described (Gray *et al.*, 1987; Bréant *et al.*, 1990, respectively). The assessment of DNA synthesis was determined by incorporation of [$^3$H]thymidine (1 μCi/ml) for the 24 h of the stimulation. Incorporation of the labeled precursor into DNA was terminated by adding three drops of 1 M ascorbic acid (Campisi *et al.*, 1982). For measurement of TCA-precipitable incorporated thymidine, cells were washed twice with cold phosphate-buffered saline (PBS), twice with cold 5% trichloroacetic acid (TCA), and solubilized in 0.3 ml of 0.1 N NaOH, 0.1% SDS. An aliquot (0.25 ml) of the solubilized cells was mixed with 5 ml of Aqualyte Scintillation Liquid (JT Baker B.V., Deventer, Holland) and counted in a LKB beta-spectrometer. All the experiments were carried out in triplicate and repeated at least twice.

### *Collection and processing of HIT cell-conditioned medium*

HIT cells grown for 3 days in the presence of serum were washed twice with PBS, trypsinized in serum-free RPMI and replated at the same cellular density in that medium. Twenty-four hours later the medium was collected, centrifuged to remove any detached cells and frozen before testing the mitogenic activity. Before the gel filtration, the conditioned medium was thawed 20 min at 37°C, lyophilized and reconstituted in 1/10 of its initial volume. One milliliter was applied onto a 1 × 60 cm Sephacryl S100 column (Pharmacia, St Quentin-en-Yvelines, France). Elution was carried out at 20°C in PBS at a flow rate of 22 ml/h. Collected fractions (1.5 ml) were frozen and their mitogenic activity was assayed on quiescent βTC1 cells at a 1/4 dilution in RPMI. The column was calibrated by sieving of molecular-weight standards: glucagon, ribonuclease A, chymotrypsinogen A, ovalbumin and Blue Dextran 2000.

Alternatively, the thawed conditioned medium was loaded on an heparin-agarose column equilibrated in RPMI 1640. After elution with 1 M NaCl, the extract was brought with water to 1/10 of the loading volume and frozen. The mitogenic activity was assayed on quiescent cells at a 1/40 dilution in RPMI for the eluted sample, at a 1/4 dilution in RPMI for the flow-through and compared to that of the initial sample.

### *Cell cycle analysis*

For cytofluorometric analysis, $5 \times 10^6$ cells were plated onto 100 mm Petri dishes in standard medium, allowed to attach for 24 h and then washed and incubated in serum-free medium for 1 or 2 days. Approximately 90% of the cells remained attached. Alternatively, control cells were treated similarly in the presence of serum. Cells were harvested with trypsin-EDTA, washed twice with PBS containing 1.5 mM EDTA and resuspended in the same buffer. Starting from $4.4 \pm 0.2 \times 10^6$ cells at the beginning of the experiment, the total number of cells was as follows: 8% FCS 48 h, $7.3 \pm 0.3 \times 10^6$ cells; 0 FCS 24 h, $4.9 \pm 0.2 \times 10^6$ cells; 0 FCS 48 h, $5.9 \pm 0.2 \times 10^6$ cells. Cells fixed by addition of ethanol to a final concentration of 70% were stored at 4°C for at least 18 h. They were then pelleted, resuspended in PBS to a final concentration of $10^6$ cells/ml and 1 mg/ml RNAse and 20 mg/ml propidium iodide were added. After 15–30 min at room temperature, the suspension was analyzed at a rate of about 500 cells/s in a flow cytometer (Orthocytofluorograph 50H, Ortho Instruments, Ortho Diagnostic Systems, Westwood, Minnesota). Excitation wavelength at 488 nm was emitted from a 3W argon laser operating at 500 mW. The instrument was standardized each day for mean channel fluorescence with fluorescent microspheres. Aggregates were gated out by using pulse shape analysis (peak *vs* area fluorescence cytograms). The red fluorescence was detected in a linear mode above 550 nm wavelength. All data were collected in a list mode and analysed with a DS1 computer (Ortho Diagnostic Systems).

### *Measurement of the release of IGF-I and II*

The amount of IGF-I and II was measured in different conditioned media after acidic gel filtration by a specific radioimmunoassay or a competitive protein binding assay, respectively (Binoux *et al.*, 1986). The day after plating, HIT cells in the serum-free medium were treated with suramin to eliminate any trace amount of growth factors. Twenty-four hours later, suramin was removed, fresh medium was added and collected 24 h thereafter for the assay of IGF-I and II. The release of IGF-I and II was also measured in the medium of quiescent βTC1 cells incubated for 3 days in the absence of serum.

### *Analysis of RNA*

For RNA extraction, cells were seeded in 100 mm Petri dishes ($5 \times 10^6$ cells/dish). The cells were then chilled on ice, washed once with cold PBS and total RNA was isolated using the LiCl/urea precipitation procedure (Auffray & Rougeon, 1980). Twenty micrograms of total RNA were denatured in 2.2 M formaldehyde for 10 min at 60°C, fractionated by electrophoresis in formaldehyde 2.2 M-agarose 1% gel, transferred onto a hybond N filter (Amersham, Les Ulis, France), U.V.-cross-linked and hybridized overnight at

42°C with $^{32}$P-labeled probes (labeled by Multiprime, Amersham, to a specific activity of $2-4\times10^9$ d.p.m./μg) in 5×SSPE, 5×Denhardt's, 0.1% SDS and 50% formamide (Maniatis *et al.*, 1982). The blots were then washed twice in 2×SSC-0.1% SDS for 15 min at room temperature, for 1 h at 45°C in the same buffer and then for 30 min in 0.1×SSC-0.1% SDS at 45°C. Blots were exposed to X-ray films (Kodak XAR5) with intensifying screens (Dupont Cronex type Quanta III, Euromedica, Paris, France) at −80°C. Exposure time varied from overnight to 3 days.

*Probes*

The probes were rat α-tubulin cDNA (Lemishka *et al.*, 1981), rat insulin cDNA (Ullrich *et al.*, 1977), BglII–PvuII fragment of FBJ provirus (v-*fos*) (Curran *et al.*, 1982), RJA1 clone corresponding to mouse c-*jun* cDNA (Ryseck *et al.*, 1988), and human c-*myc* cDNA (Dalla Favera *et al.*, 1982). We are grateful to P. Nurse for making us available the mouse cdc2 cDNA and to G. Christofori for the mouse betacellulin cDNA (Shing *et al.*, 1993). The probes encoding human IGF-I (Le Bouc *et al.*, 1986) and IGF-II cDNA (Le Bouc *et al.*, 1987) were kindly provided by Y. Le Bouc in agreement with Transgene.

*Statistical methods*

Table 1 presents means ± SEM of three independent experiments. The overall analysis of variance (F test) within each phase of the cell cycle was not significantly different for the three culture conditions.

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