Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 5936–5940, August 1988
Cell Biology

Autocrine growth induced by the insulin-related factor in the insulin-independent teratoma cell line 1246-3A

(autostimulatory growth factor/proliferation/secretion/binding)

YUKIO YAMADA AND GINETTE SERRERO*

W. Alton Jones Cell Science Center, 10 Old Barn Road, Lake Placid, NY 12946

Communicated by Gordon H. Sato, December 18, 1987

ABSTRACT **An insulin-independent teratoma-derived cell line, called 1246-3A, has been isolated from the adipogenic cell line 1246, which stringently requires insulin for proliferation. The 1246-3A cell line, which can proliferate in the absence of exogenous insulin, produces in its conditioned medium a growth factor similar to pancreatic insulin by its biological and immunological properties. This factor, called "insulin-related factor" (IRF), was purified and iodinated to study its binding to cell surface receptors. ^{125}I-labeled IRF binding to intact 1246-3A cells is lower than to 1246 cells. Cell surface binding can be restored by culturing the 1246-3A cells in the presence of an anti-porcine insulin monoclonal antibody or by acid prewash of the cells prior to performing the binding. Scatchard analysis of binding indicates that IRF secreted by the 1246-3A cells partially occupies high-affinity binding sites on the producer cells. Moreover, insulin monoclonal antibody inhibits the proliferation of the IRF-producing 1246-3A cells, suggesting that these cells are dependent on the secreted IRF for growth in culture. We conclude that the insulin-related factor secreted by the insulin-independent 1246-3A cells stimulates their proliferation in an autocrine fashion.**

1246 cell line is an adipogenic cell line derived from a C3H mouse teratoma. This cell line has the ability to proliferate and differentiate in defined medium (1) and stringently requires insulin to undergo both processes (2). From 1246 cells maintained in defined medium deprived of insulin, an insulin-dependent cell line called 1246-3A was isolated (3) and cloned. Unlike the parent cell line, the 1246-3A cell line can proliferate in the absence of insulin, has lost the ability to differentiate, and produces tumors when injected into syngeneic hosts, and its conditioned medium contains factor able to stimulate the growth of the parent cell line maintained in the absence of insulin (3). Gel filtration of 1246-3A-conditioned medium on Sephadex G-50 in acidic conditions resolved the growth-promoting activity into two peaks: one was eluted in the void volume, and the other migrated with an apparent molecular mass of 6 kDa (4). The 6-kDa factor presented immunological and biochemical similarities to pancreatic insulin and was distinct from insulin-like growth factors I and II (IGF-I and IGF-II) by radioimmunoassay (4). Because of its similarities with insulin, the 6-kDa factor was called insulin-related factor (IRF). IRF was purified from 1246-3A-conditioned medium by a three-step purification procedure, including ion-exchange chromatography, immunoaffinity chromatography, and reverse-phase HPLC (5). Pure IRF was similar to pancreatic insulin by its biochemical properties and by its amino acid composition (5). As the 1246-3A cells that produced IRF were independent of the presence of exogenous insulin for proliferation, we examined whether this was due to the fact that IRF acted as an autostimulatory growth factor for the producer cells. Experiments investigating the binding of radiolabeled insulin to intact 1246 and 1246-3A cells had shown that insulin-specific binding on the insulin-independent variant cells was lower than on the 1246 cells but could be restored to a value equivalent to the one found on 1246 cells by prewashing the cells with a buffer at pH 5.5 (4). These data, in support of the autocrine hypothesis of Sporn and Todaro (6), indicated indirectly that ectopic IRF secreted by the 1246-3A cells in their culture medium partially occupied their insulin binding sites. Since pure IRF could be obtained from 1246-3A-conditioned medium (5), it was possible to conclusively examine the hypothesis that IRF stimulated the 1246-3A cell growth in an autocrine fashion by: (*i*) comparing IRF binding on the 1246 and 1246-3A cells, (*ii*) determining if secreted IRF could occupy cell surface receptors on the 1246-3A cells, and (*iii*) examining if 1246-3A growth stimulation can be inhibited when IRF is removed by antibodies.

MATERIALS AND METHODS

Dulbecco's modified Eagle's medium (DME medium), Ham's F-12 nutrient mixture, RPMI 1640 medium, and trypsin were purchased from GIBCO. Disposable tissue culture plastic ware was from Falcon. Fetal bovine serum was from HyClone (Logan, UT), and Hepes was obtained from Research Organics (Cleveland). Na^{125}I was from New England Nuclear. Protein A MAPS II kit (monoclonal antibody preparative system) was from Bio-Rad. Human plasma fibronectin and bovine pituitary fibroblast growth factor were purchased from Collaborative Research (Waltham, MA). Insulin, transferrin, and all other chemicals were obtained from Sigma.

Cell Culture. Stock culture of 1246 cells were performed in 1:1 (vol/vol) DME medium/Ham's F-12 medium (DME/F-12) supplemented with 10% fetal bovine serum. 1246-3A cells were maintained in 3F medium: DME/F-12 medium supplemented with fibronectin (2.5 μg/ml), transferrin (10 μg/ml), and fibroblast growth factor (25 ng/ml) as described (3).

Purification and Iodination of IRF. IRF was purified to homogeneity from 1246-3A-conditioned medium by a three-step purification procedure, including ion-exchange chromatography on Amberlite CG50, immunoaffinity chromatography on a column of Affi-Gel 10 coupled with anti-porcine-insulin monoclonal antibody and reverse-phase HPLC on octadecyl support. The details of these purification procedures have been described elsewhere (5). IRF eluted from the HPLC column was iodinated by the chloramine-T method established for insulin (7). The labeling reaction was stopped by adding excess tyrosine instead of sodium metabisulfite. The specific activity of ^{125}I-labeled IRF (^{125}I-IRF) was 110 μCi/μg (1 Ci = 37 GBq).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: IRF, insulin-related factor; IGF-I and -II, insulin-like growth factors I and II.

*To whom reprint requests should be addressed.

Purification of an Anti-Insulin Monoclonal Antibody. A hybridoma cell line producing anti-insulin monoclonal antibody, called 4B9, was used (5). Supernatant culture medium from a variant of this cell line was used to purify anti-insulin monoclonal antibody. A serum-free culture supernatant of the hybridoma cell line was used as the starting material to avoid contamination of the antibody preparation with serum proteins. In addition, because defined media generally used to support the growth of hybridoma cell lines in serum-free conditions contain insulin (8), we isolated a variant of the hybridoma cell line 4B9 that was able to proliferate in defined medium in the absence of insulin. Such a variant hybridoma cell line, called 4B9-I, has the ability to proliferate in a defined medium referred to as "TES-RDF," consisting of a mixture of RPMI 1640, DME medium, and F-12 media, 1:2:2 (vol/vol), supplemented with transferrin (10 μg/ml), ethanolamine (20 μM), and selenium (1 nM). Briefly, 4B9-I cells were cultured in TES-RDF medium. Medium from confluent cells was collected every 3 days and replaced by fresh medium. The IgG was purified from the hybridoma-conditioned medium by using the protein A MAPS II kit from Bio-Rad according to the manufacturer's specifications. Recombinant human IGF-I from Amgen Biologicals (Thousand Oaks, CA) and rat IGF-II at concentrations up to 1 μg/ml did not cross-react with the anti-insulin monoclonal antibody as determined by insulin radioimmunoassay. Anti-IGF-II monoclonal antibody was a gift from Katsuzo Nishikawa (Kanazawa Medical University, Kanazawa, Japan).

^{125}I-IRF Binding Assay. 1246 and 1246-3A cells were inoculated in DME/F-12 medium supplemented with 3% (vol/vol) fetal bovine serum at a cell density of 2×10^3 cells per cm^2 in Linbro multiwell plates (9 cm^2). When the cells reached confluency, they were washed twice with DME/F-12 medium and then cultivated in DME/F-12 medium in either the absence or the presence of anti-insulin or anti-IGF monoclonal antibodies for 4 days. The medium was changed every 2 days. After the incubation time, the wells were washed twice with DME/F-12 medium containing 0.1% bovine serum albumin at pH 7.4. This was followed by three washes with phosphate-buffered saline either at pH 7.4 or at pH 5.0 in the presence of 0.1% bovine serum albumin as described (4). Binding of ^{125}I-IRF was then performed by using 2×10^5 cpm of ^{125}I-IRF (110 μCi/μg) in the binding buffer as described (4). Nonspecific binding was determined in the presence of 10 μg of bovine insulin per ml.

Scatchard Analysis of IRF Binding to 1246-3A Cells. 1246-3A cells were cultivated as described in the previous paragraph. At the time of binding, the 1246-3A cells were washed three times with either the pH 7.4 or the pH 5.0 buffer. Binding of iodinated IRF (4×10^4 cpm; 110 μCi/μg) was performed in the presence of increasing concentrations of unlabeled IRF at 4°C for 4 hr as described above. Nonspecific binding was determined in the presence of 10 μg of unlabeled bovine insulin per ml.

Effect of Anti-Insulin and Anti-IGF-II Monoclonal Antibodies on the Proliferation of 1246-3A Cells in Defined Medium. *Experiment 1.* 1246-3A cells were inoculated at a density of 3.0×10^3 cells per cm^2 in 24-well dishes in DME/F-12 medium supplemented with transferrin (10 μg/ml) and fibronectin (2.5 μg/ml) in the absence or presence of increasing concentrations of protein-purified anti-insulin IgG. IgG was added every 2 days in the culture medium. The cell number was determined at day 4 as described (4).

Experiment 2. 1246-3A cells were inoculated under the same conditions as the ones described in experiment 1 but in either the absence or the presence of 0.3 μM anti-insulin IgG, added every 2 days. Cells from quadruplicate dishes were counted every day by a Coulter Counter.

RESULTS

Binding of Labeled IRF to 1246 and 1246-3A Cells. Binding of labeled IRF to monolayers of 1246 cells and 1246-3A cells was performed under different conditions: (*i*) after the cells had been washed with a buffer at pH 7.4; (*ii*) after the cells had been washed with a buffer at pH 5.0, which is known to dissociate excess growth factors bound to cell surface receptors (8); (*iii*) on cells that had been cultured either in the presence of an anti-insulin monoclonal antibody or an anti-IGF-II monoclonal antibody [preliminary experiments had shown that incubation of partially purified IRF with an anti-insulin monoclonal antibody (0.3 μM) could bind and immunoprecipitate IRF, whereas similar incubation with an anti-IGF-II monoclonal antibody did not have any effect]; and (*iv*) on cells cultured with the monoclonal antibodies and prewashed with the buffer at pH 5.0. ^{125}I-IRF bound 62% less to 1246-3A cells than to 1246 cells after they had been washed with a buffer at pH 7.4 (Fig. 1). When the cells were prewashed with a buffer at pH 5.0 that is known to dissociate excess growth factors specifically bound to cell surface, the binding of IRF to 1246-3A cells increased 2-fold. Acid prewash had no effect on IRF binding to 1246 cells. This could be explained by the fact that 1246 cells do not produce IRF (data not shown). IRF binding to 1246-3A cells also was observed when the cells were cultivated for 4 days in the presence of anti-insulin monoclonal antibody known to bind IRF. In contrast, the binding profile of 1246-3A cells cultured with anti-IGF-II monoclonal antibody were identical to that of the control. When the cells that had been cultivated in the presence of the anti-insulin monoclonal antibody were

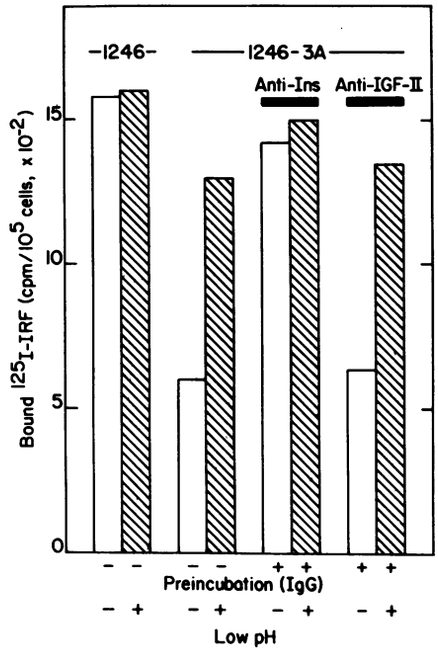

FIG. 1. Effect of prewash and culture in the presence of anti-insulin monoclonal antibody on ^{125}I-IRF binding to 1246 and 1246-3A cells. 1246 or 1246-3A cells were inoculated in DME/F-12 medium containing 3% fetal bovine serum until they reached confluency. They were washed with DME/F-12 medium for 24 hr before being submitted to different culture conditions: culture in the absence (−) or presence (+) of the IgG fraction containing anti-insulin monoclonal antibody (Anti-Ins) or anti-IGF-II monoclonal antibody (Anti-IGF-II). Prior to binding, the cells were washed with phosphate-buffered saline containing 0.1% bovine serum albumin at pH 5.0 (low pH +; hatched bars) or at pH 7.4 (low pH −; open bars) as described. Specific binding was calculated by subtracting nonspecific binding in the presence of 10 μg of bovine insulin per ml from the total binding.

washed at pH 5.0 prior to performing the binding, IRF binding to 1246-3A cells was the highest and became identical to IRF binding to the 1246 cells. Thus, treatments allowing either dissociation of IRF from the cell surface (acid prewash) or prevention of IRF binding to it (incubation with anti-insulin monoclonal antibody) resulted in restoring ^{125}I-IRF binding on the producer cells to a level similar to the one observed on the 1246 cells. These data would suggest that ectopic IRF, once secreted by the 1246-3A cells in their culture medium, partially occupies their binding sites on the same cell, thus partially preventing the binding of exogenously added IRF. To investigate this possibility, ^{125}I-IRF binding to 1246-3A cells was examined.

Scatchard Analysis of IRF Binding on 1246-3A Cells. Binding of radioiodinated IRF was performed on 1246-3A cells in the presence of increasing concentrations of nonradioactive IRF after they had been prewashed with a buffer either at pH 7.4 or at pH 5.0. The binding data were analyzed by the method of Scatchard (Fig. 2). In both cases, the Scatchard representations were curvilinear, indicating the presence of two classes of IRF binding sites. The number of binding sites and equilibrium dissociation constants (K_d) were determined from the plots (Fig. 2). The cells that were prewashed with the buffer at pH 7.4, which does not dissociate prebound IRF from the 1246-3A cells, showed a lower number of high-affinity binding sites than did the cells washed at pH 5.0. However, the number of sites and the K_d value for the low-affinity binding sites were similar to those of the cells prewashed at pH 5.0. Prewash of the 1246-3A cells with the buffer at pH 5.0, known to dissociate IRF bound to the cell surface, resulted in a 2-fold increase in the number of high-affinity binding sites without changing the K_d value (Fig. 2 *Inset*). The number of high-affinity binding sites and the K_d value measured in these conditions (pH 5.0 wash) were similar to the ones determined for the 1246 cells, which did not secrete IRF (Table 1). This experiment indicates that IRF secreted by the 1246-3A cells in their culture medium partially occupies the high-affinity binding sites on the producer cells.

Anti-Insulin Monoclonal Antibody Blocks the Proliferation of 1246-3A Cells. We then examined whether the secreted IRF, once bound to the 1246-3A cells, could stimulate the producer cell proliferation via an autocrine mechanism. For this purpose, we determined whether the growth of the producer cells could be inhibited if the secreted IRF were

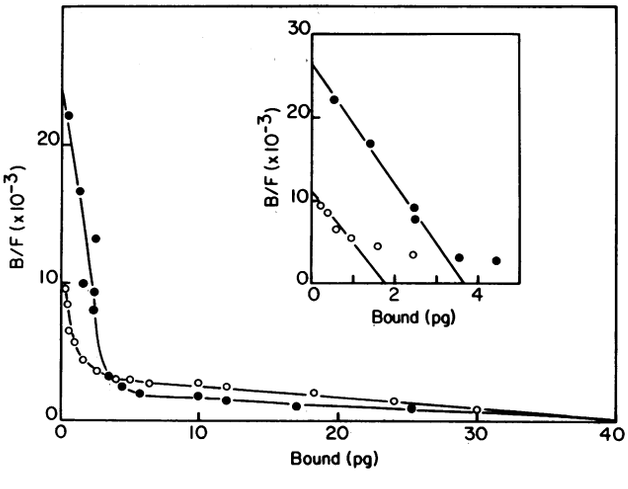

FIG. 2. Scatchard representation of ^{125}I-IRF binding to 1246-3A cells. Prior to binding, the cells were washed several times with a solution of 0.1% bovine serum albumin in phosphate-buffered saline at pH 7.4 (○) or pH 5.0 (●). (*Inset*) Larger scale representation of the high-affinity binding. B/F, bound/free.

Table 1. High-affinity binding sites and dissociation constants for IRF binding on 1246 and 1246-3A cells

Cell line	High-affinity receptors	
	K_d, M × 10^{10}	Sites per cell
1246	2.9	7500
1246-3A		
Acid wash	2.3	7300
Neutral wash	2.6	3500

^{125}I-IRF binding was performed as described in the legend of Fig. 2. The number of sites per cell and K_d values were calculated from a Scatchard plot of IRF binding to 1246 and 1246-3A cells.

prevented from binding to the 1246-3A cells. To investigate this possibility, the 1246-3A cells were cultivated in defined medium, in the presence of increasing concentrations (1 nM to 1 μM) of the anti-insulin monoclonal antibody, which is known to bind to IRF and prevent its binding to the cells. Cell number was determined after 4 days in culture and compared to the number of cells maintained in the defined medium in the absence of the anti-insulin IgG. 1246-3A cell growth was inhibited by the addition in the culture medium of anti-insulin monoclonal antibody (Fig. 3*a*). An inhibition of 50% as compared to the control was obtained at a concentration of anti-insulin monoclonal antibody equal or superior to 0.1 μM. The growth of 1246-3A cells cultivated in defined medium was measured in the absence and presence of 0.3 μM anti-insulin IgG. Fig. 3*b* indicates that the presence of the anti-insulin monoclonal antibody in the culture medium decreased the growth rate of the producer cells. Doubling time was 48 hr in the presence of the anti-insulin monoclonal antibody and 24 hr in its absence. Cultivation of the 1246-3A cells in defined medium containing increasing concentrations of anti-IGF-II IgG did not affect the cell proliferation (Fig. 4). These data directly show that growth was impaired if IRF secreted in the medium was prevented from binding to the 1246-3A cells. Based on these observations, it was concluded that IRF secreted by the 1246-3A cells was required to support their growth in defined medium and acted in an autocrine fashion.

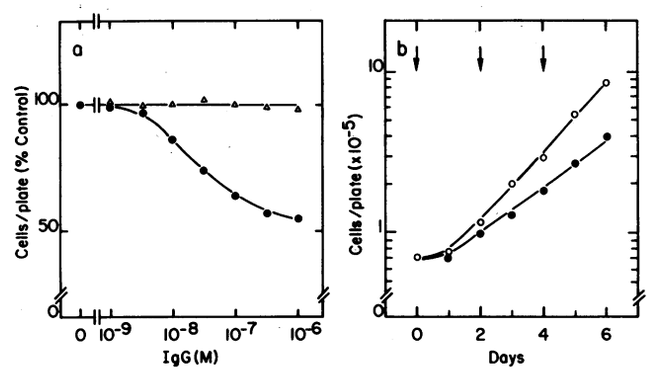

FIG. 3. Effect of culture in the presence of anti-insulin monoclonal antibody on 1246-3A cell proliferation. (*a*) 1246-3A cells (3 × 10^3 cells per cm^2) were inoculated in DME/F-12 medium supplemented with fibronectin (2.5 μg/ml) and transferrin (10 μg/ml), in the absence (△) or presence (●) of increasing concentrations of anti-insulin monoclonal antibody (IgG fraction) added at day 0 and day 2. Cell number was determined at day 4. Values were expressed as the percentage of control, calculated as the number of cells cultivated in the presence of the IgG fraction divided by the number of cells cultivated in its absence. (*b*) 1246-3A cells (3 × 10^3 cells per cm^2) were inoculated in 35-mm dishes in the defined medium described above in the absence (○) or presence (●) of 0.3 μM anti-insulin monoclonal antibody (IgG fraction) added every 2 days. Cell numbers from duplicate dishes were measured every day as described. The arrows indicate the time when anti-insulin IgG was added.

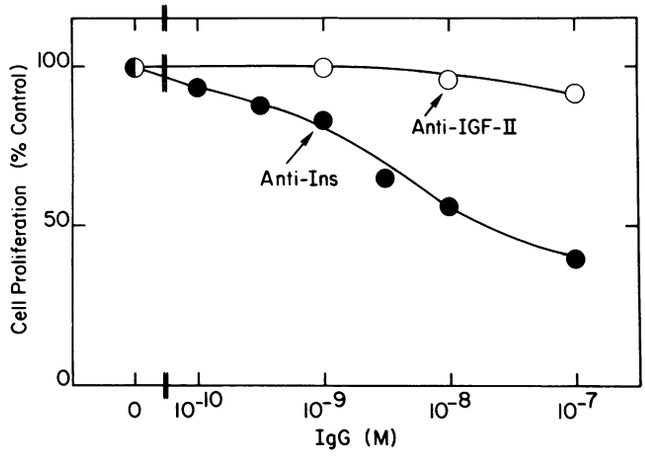

FIG. 4. Comparative effect of anti-insulin monoclonal antibody and anti-IGF-II monoclonal antibody on 1246-3A cell proliferation. Culture conditions were the same as the ones described in the legend of Fig. 3. 1246-3A cells were cultivated either in the presence of anti-insulin monoclonal antibody (●; anti-Ins) or in the presence of anti-IGF-II monoclonal antibody (○; anti-IGF-II). Cell number was determined at day 4.

CONCLUSION

The insulin-independent cell line 1246-3A has been isolated by maintaining the teratoma-derived adipogenic cell line 1246 in insulin-free medium. 1246-3A cells can proliferate in a defined medium deprived of insulin, whereas the parent cell line 1246 stringently requires insulin to proliferate (3). We have demonstrated earlier that the 1246-3A cells produce a polypeptide growth factor structurally and biochemically similar to pancreatic insulin and different from IGF-I and IGF-II, which we are tentatively calling IRF (insulin-related factor). Experiments described in the present paper provide conclusive evidence that IRF acts as an autocrine growth factor for the 1246-3A cells. ^{125}I-IRF binding is lower on the 1246-3A cells than on the 1246 cells. The binding can be restored both by washing the cells in acidic conditions that dissociate growth factor already bound to cell surface receptors and by culturing the cells in the presence of an anti-insulin monoclonal antibody. Scatchard analysis of the binding shows that IRF secreted in the culture medium occupies high-affinity binding sites on the producer cells. It is probable that IRF occupies high-affinity insulin receptors on the 1246-3A cells. Several pieces of evidence support this hypothesis: (*i*) ^{125}I-labeled insulin binding to 1246-3A cells is 50% lower than on 1246 cells and can be restored by acid wash of the cells prior to performing the binding (4); (*ii*) pure IRF can interact directly with insulin receptors and stimulate tyrosine kinase activity of insulin receptors on both cell lines (H. Gazzano, Y.Y., and G.S., unpublished data) and; (*iii*) binding parameters determined for IRF are similar to the ones determined for insulin binding on 1246 and 1246-3A cells prewashed under acidic conditions. Therefore, it is likely that the secreted IRF interacts directly with insulin receptors on the producer cells and acts as an autostimulatory growth factor. This latter conclusion is based upon the finding that anti-insulin monoclonal antibody inhibited the proliferation of 1246-3A cells, presumably by binding the secreted IRF present in the culture medium and preventing it from interacting with the cell surface receptors on 1246-3A cells. The fact that culturing the cells in the presence of anti-IGF-II monoclonal antibody did not impair their growth excluded the possibility that the anti-insulin antibody was inhibiting cell growth in a nonspecific manner or by being toxic to the cells. Although anti-insulin antibody inhibited cell growth, the highest inhibition obtained was 50%, even with excess concentrations of antibody. One possible explanation is that the 1246-3A cells synthesize and secrete in their conditioned medium another type of growth factor, distinct from IRF, which does not interact with anti-insulin antibody and does not compete with insulin for binding on 1246 cells (4). It is possible that this other growth factor, produced in the conditioned medium, is also required to maximally autostimulate the growth of 1246-3A cells.

Autocrine stimulation of growth has been demonstrated for several cell types, generally transformed or tumorigenic, producing hormones or growth factors (9–13). For normal cells, it has been postulated that at the early stages of embryonic development, cell functions are regulated by an autocrine process (6). Teratocarcinoma cells, which offer an *in vitro* system to study early events involved in embryonic development, have been shown to secrete several known hormones and growth factors (14–21). In some cases, autocrine regulation of growth has been demonstrated (14, 15). The present paper indicates that an IRF produced by a teratoma-derived cell line acts as an autocrine growth factor. As several hormones and growth factors produced by teratocarcinoma and teratoma cells also have been found in the embryo (22–24), it is possible to assume that the autocrine growth-stimulatory effect of insulin demonstrated with the teratoma-derived cell line may represent a physiological regulatory control during embryonic development. Such a role for insulin has been postulated by de Pablo *et al.*, who demonstrated that immunoreactive insulin was found in prepancreatic 2-day-old chicken embryos (25) and was required for their development (26). Studies with the insulin-independent variant 1246-3A cell line should make it possible to investigate the putative existence of embryonic insulin in mammalian species.

We thank Dr. Katsuzo Nishikawa for the gift of anti-IGF-II monoclonal antibody, Mrs. Julie Lamb for preparing the manuscript, and Marina LaDuke for the illustrations. This work was supported by grants from the Juvenile Diabetes Foundation File (185221) and by National Institutes of Health Grant 1-PO1-CA 37589.

1. Serrero, G. & Khoo, J. C. (1982) *Anal. Biochem.* **120,** 351–359.
2. Serrero, G. (1983) in *Hormonally Defined Media: A Tool in Cell Biology*, eds. Fischer, G. & Wieser, R. J. (Springer, Berlin), pp. 366–376.
3. Serrero, G. (1985) *In Vitro Dev. Cell Biol.* **21,** 537–540.
4. Yamada, Y. & Serrero, G. (1986) *Biochem. Biophys. Res. Commun.* **135,** 533–540.
5. Yamada, Y. & Serrero, G. (1987) *J. Biol. Chem.* **262,** 209–213.
6. Sporn, M. B. & Todaro, G. J. (1980) *N. Engl. J. Med.* **303,** 878–880.
7. Freychet, P., Roth, J. & Neville, D. M., Jr. (1971) *Biochem. Biophys. Res. Commun.* **43,** 400–408.
8. Haigler, H. T., Maxfield, F. R., Willingham, M. C. & Pastan, I. (1980) *J. Biol. Chem.* **255,** 1239–1241.
9. Adkins, B., Leutz, A. & Graf, T. (1984) *Cell* **39,** 439–445.
10. Huang, J. S., Huang, S. S. & Deuel, T. F. (1984) *Cell* **39,** 79–87.
11. Betsholtz, C., Westermark, B., Ek, B. & Heldin, C. H. (1984) *Cell* **39,** 447–457.
12. Kaplan, P. L., Anderson, M. & Ozanne, B. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 485–489.
13. Cuttitta, F., Carney, D. N., Mulshine, J., Moody, T. W., Fedorko, J., Fischler, A. & Minna, J. D. (1985) *Nature (London)* **316,** 823–826.
14. Evain-Brion, D., Binet, E., Donnadieu, M., Laurent, P. & Anderson, W. B. (1984) *Dev. Biol.* **104,** 406–412.
15. Binet, E., Laurent, P. & Evain-Brion, D. (1985) *J. Cell. Physiol.* **124,** 288–292.
16. Rizzino, A., Orme, L. S. & DeLarco, J. E. (1983) *Exp. Cell. Res.* **143,** 143–152.
17. Heath, J. K. & Isacke, C. M. (1984) *EMBO J.* **3,** 2957–2962.
18. Rizzino, A. & Bowen-Pope, D. (1985) *Dev. Biol.* **110,** 15–22.
19. Gudas, L. J., Singh, J. P. & Stiles, C. D. (1983) in *Teratocar-*

cinoma Stem Cells, Cold Spring Harbor Conferences on Cell Proliferation, eds. Silver, L. M., Martin, G. R. & Strickland, S. (Cold Spring Harbor Lab., Cold Spring Harbor, NY), Vol. 10, pp. 229–236.
20. Anderson, W. B., Nagarajan, L., Jetten, A., Rechler, M. M. & Nissley, P. (1983) *Cell. Biol. Int. Rep.* **7,** 563.
21. Heath, J. K. & Isacke, C. M. (1983) *Cell. Biol. Int. Rep.* **7,** 561–562.
22. Adamson, E. D. (1982) *Dev. Biol.* **91,** 227–234.
23. d'Ercole, A. J., Applewhite, G. T. & Underwood, L. E. (1980) *Dev. Biol.* **75,** 315–328.
24. Rizzino, A. (1985) *In Vitro Dev. Cell Biol.* **21,** 531–536.
25. de Pablo, F., Roth, J., Hernandez, E. & Pruss, B. B. (1982) *Endocrinology* **111,** 1909–1914.
26. de Pablo, F., Girbau, M., Gomez, J., Hernandez, E. & Roth, J. (1985) *Diabetes* **34,** 1063–1066.