Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 11217–11221, December 1993
Cell Biology

Simian virus 40 large tumor antigen is unable to transform mouse embryonic fibroblasts lacking type 1 insulin-like growth factor receptor

CHRISTIAN SELL*, MICHELE RUBINI*, RAPHAEL RUBIN*, JEH-PING LIU†, ARGIRIS EFSTRATIADIS†, AND RENATO BASERGA*‡

*Jefferson Cancer Institute, Thomas Jefferson University, Philadelphia, PA 19107-5541; and †Department of Genetics and Development, Columbia University, New York, NY 10032

Communicated by I. S. Edelman, August 26, 1993 (received for review August 2, 1993)

ABSTRACT **Fibroblast cell lines were established from mouse embryos homozygous for a targeted disruption of the *Igf1r* gene, encoding the type 1 receptor for insulin-like growth factor I (IGF-I) and from their wild-type littermates. The cells from the wild-type embryos (W cells) grow in serum-free medium supplemented with platelet-derived growth factor, epidermal growth factor, and IGF-I, whereas the cells from *Igf1r*(−/−) embryos (R^- cells) do not, although they grow at a reduced rate in 10% fetal calf serum. The simian virus 40 (SV40) large T antigen, expressed from a transfected plasmid, can transform W cells, which form foci in monolayer cultures and colonies in soft agar (anchorage-independent growth). In contrast, the SV40 large tumor antigen, although normally expressed from the transfected template, is unable to transform R^- cells, which remain contact-inhibited and fail to grow in soft agar. The transformed phenotype is restored if the R^- cells carrying the SV40 large tumor antigen are stably transfected with a plasmid expressing the human IGF-I receptor. These results demonstrate that signaling via the IGF-I receptor is an indispensable component of the SV40 transformation pathway. This conclusion is further supported from the results of antisense RNA experiments with tumor cell lines showing that interference with the function of the IGF-I receptor has a profound effect on anchorage-independent growth, even under conditions that only modestly affect growth in monolayers.**

Interaction of the insulin-like growth factor type I (IGF-I) receptor (IGF-IR) with its ligands (IGF-I, IGF-II, and insulin at supraphysiological concentrations) plays a pivotal role in embryonal development (1) and in the proliferation of several types of cells in culture (2–5). The simian virus 40 (SV40) large tumor antigen (TAg) is one of the most effective transforming agents of mouse cells in culture (6, 7), lowering the growth factor requirements and inducing the ability to grow in soft agar (6, 8–11). Recently, SV40 TAg was shown to lower the growth-factor requirements of 3T3 cells and abrogate the requirement for IGF-I by increasing expression of endogenous IGF-I (12). These results were confirmed in human fibroblasts immortalized by SV40 TAg (13). Thus, whether the IGF-IR pathway is also involved in other parameters of transformation, beyond its ability of lowering growth-factor requirements, is an interesting question. To address this question directly, we have developed fibroblast-like cell lines from mouse embryos homozygous for a targeted null mutation of the *Igf1r* gene (1), designated R^- cells, and then derived from R^- cells stable transfectants that express SV40 TAg. Here we show that, in contrast to control cell lines from littermate wild-type embryo fibroblasts, SV40 TAg cannot transform cells lacking a functional IGF-IR. This result shows that, in mouse embryo fibroblasts in culture, function of the IGF-IR is a prerequisite for operation of the SV40 TAg pathway.

MATERIALS AND METHODS

Cell Cultures. Mouse embryos were dissected from anesthetized females at day 18 of gestation and genotyped by Southern analysis as described (1), using DNA prepared from their tails. Wild-type and homozygous *Igf1r*(−/−) mutant littermates were used to establish primary cultures of embryonic fibroblasts as described (14). Briefly, the embryos were minced, and after treatment with trypsin for 15 min, the cells of the resulting suspension were plated onto 100-mm culture dishes and cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO/BRL)/10% fetal bovine serum. The cultures were maintained at subconfluent levels by treating with trypsin every 3 days and reseeding at a density of 1.5×10^3 cells per cm^2, following the same protocol used to generate 3T3 cell lines (15). Primary cultures underwent crisis after 2–4 weeks in culture. R^- cultures entered crisis later than the wild-type cells due to the relatively slow doubling rate.

T98G cells (16) are a human glioblastoma cell line that produces large amounts of IGF-I. Two other cell lines were generated from T98G cells, expressing, respectively, a sense and an antisense RNA to the human IGF-IR RNA. Preparation of the expression plasmids is described elsewhere (D. Ambrose, M. Resnicoff, D. Coppola, C.S., M. Miura, S. Jameson, R.B., and R.R., unpublished work); the expressed fragment is 309 bp long. C6 (17, 18) is a rat glioblastoma cell line, already used extensively in several laboratories.

Plasmid Transfection. Cells were transfected as described (12) with DNA of the following plasmid constructs: (*i*) ptsA58H (12), which contains both hygromycin-resistance gene (*hyg*) and sequence encoding tsA58, a temperature-sensitive SV40 TAg (19); (*ii*) pSV2G (20), which contains sequence encoding the wild-type SV40 TAg and which was cotransfected with a plasmid containing the hygromycin resistance gene (LHL4) (21); and (*iii*) Cvn-IGF-IR, which contains both the neomycin-resistance gene (*neo*) and the entire coding sequence of human IGF-IR cDNA, both under the control of SV40 promoter (22, 23).

Primary cultures were extremely sensitive to hygromycin, and selection was done at a drug concentration of 10 μg/ml. Cell proliferation in anchorage-dependent conditions was

Abbreviations: IGF-I, insulin-like growth factor I; IGF-IR, IGF-I receptor; SV40, simian virus 40; TAg, large tumor antigen; PDGF, platelet-derived growth factor.

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.

‡To whom reprint requests should be addressed at: Jefferson Cancer Institute, 624 Bluemle Life Sciences Building, 233 South 10th Street, Philadelphia, PA 19107-5541.

assayed by treating the cells with trypsin and counting in triplicate every 24 hr, using a hemocytometer.

Immunostaining for TAg. Cells fixed in cold methanol were incubated with a 1:10 dilution of anti-TAg antibody (PAb 419; Oncogene Science) and then stained with a 1:100 dilution of a fluoresceinated goat antimouse IgG antibody (Oncogene Science). Staining intensity was measured in arbitrary units by computer analysis of photographic images of the stained cells.

Soft-Agar Assay. Anchorage-independent growth was assayed by scoring the number of colonies formed in 0.2% agarose (with either a 1% or 0.4% agarose underlay). TAg-transfected cells grew for 3 weeks, whereas glioblastoma cell colonies were counted after 2 weeks, due to the higher growth rate of these cells relative to TAg-transformed cells.

Cross-Linking of IGF-IR. Radioiodinated IGF-I was cross-linked to the IGF-IR, as described (24) by using disuccinimidyl suberate. After cross-linking, the proteins were resolved on an 8% polyacrylamide gel, and the dried gel was exposed to x-ray film (Kodak X-Omat) for autoradiography.

IGF-IR Autophosphorylation. Receptor autophosphorylation was measured as described (23) by using an antibody to the β subunit of the mouse IGF-IR, prepared by immunization of rabbits against a 16-mer synthetic peptide (Glu-Leu-Glu-Met-Glu-Leu-Glu-Met-Glu-Pro-Glu-Asn-Ile-Gly-Glu-Leu) coupled to keyhole limpet hemocyanin. The cellular extract was immunoprecipitated with the antibody to the IGF-IR; the precipitated proteins were resolved on an 8% polyacrylamide gel and electroblotted to nitrocellulose filters for immunoblot analysis using an antiphosphotyrosine monoclonal antibody (Upstate Biotechnology), which was visualized with the ECL system (Amersham).

Antisense Oligonucleotides. The antisense and sense oligodeoxynucleotides to the IGF-IR mRNA used for the colony-formation assay of C6 cells have been described (23). They correspond to the 18 bp following the ATG of the IGF-IR cDNA. Antisense oligonucleotides were added to the cells at seeding at a concentration of 80 μg/ml.

RESULTS

Characterization of Cultured Embryonic Cells. Cells from wild-type and homozygous *Igf1r*(−/−) mutant embryos were established initially as primary cultures and subsequently as postcrisis cell lines, which will be referred to as parental lines W and R$^-$, respectively. These lines, derived by a protocol used to generate 3T3 cells (15), have a fibroblast-like appearance. In serum-free medium supplemented with platelet-derived growth factor (PDGF) (5 ng/ml), epidermal growth factor (20 ng/ml), and IGF-I (20 ng/ml), W cells grow well, whereas R$^-$ cells fail to increase in number. Growth of R$^-$ cells can be sustained in 10% serum, but their growth rate is only 40–50% that of W cell controls (Table 1); growth of W cells under the conditions of Table 1 is essentially the same as that of BALB/c 3T3 cells.

Previously, indirect biochemical analysis showed that a functional IGF-IR is absent from primary cultures of cells isolated from day 14.5 *Igf1r*(−/−) mutant embryos (1). To confirm this result with a specific antibody and establish unequivocally that R$^-$ cells are completely devoid of IGF-IR, we performed the following experiment. After incubation with IGF-I for ligand-activated autophosphorylation of the IGF-IR, R$^-$ cells and control W cells were lysed, and a polyclonal antibody against the β subunit of mouse IGF-IR was added to immunoprecipitate any functional IGF-IR in the lysate. The precipitated proteins were solubilized in the presence of 2-mercaptoethanol, resolved electrophoretically, and transferred to a nitrocellulose membrane. The β subunit of IGF-IR, autophosphorylated in an IGF-I-dependent fashion, was visualized by immunostaining with an antiphosphotyrosine antibody (Upstate Biotechnology) and recognized by size and by response to IGF-I. The apparent molecular masses of α and β subunits of IGF-IR, resolved electrophoretically after disulfide-bond reduction and denaturation, are 135 kDa and 97 kDa, respectively. Fig. 1*A* shows that the autophosphorylated β subunit (a 97-kDa species) was detected, after IGF-I stimulation, in W cells (lane 2) but was not detected in R$^-$ cells (lane 4).

Table 1. Growth of W and R$^-$ cells in culture

Cell type	Supplements	Doublings, no.
W cells	PDGF, EGF, IGF-I	1.5
	10% serum	3.0
	Serum-free medium	0
R$^-$ cells	PDGF, EGF, IGF-I	0
	10% serum	1.5
	Serum-free medium	0

Cells were seeded at 5×10^3 cells per cm^2 in plastic dishes in DMEM medium/10% fetal calf serum. After 24 hr, growth medium was removed, cells were washed several times with Hanks' solution, and DMEM was added with the indicated supplements. Cells were counted at 48 and 72 hr after changing to the indicated condition. Number of doublings shown is that obtained at 72 hr. Repeated experiments yielded similar results. EGF, epidermal growth factor.

For confirmation, we looked for the presence of the α subunit of IGF-IR in both cell types by cross-linking radioiodinated IGF-I to cell membranes, electrophoretic analysis, and autoradiography (24). A labeled 135-kDa protein was easily detectable in W cells; this signal could be eliminated by inhibiting the radioiodinated ligand with a 1000-fold excess of unlabeled IGF-I (Fig. 1*B*, lanes 1 and 2). In contrast, it was not possible to detect a labeled protein species of this size in R$^-$ cells, even after significant autoradiogram overexposure (Fig. 1*B*, lane 3).

Assays for Cell Transformation with SV40 TAg. Parental W and R$^-$ cells were transfected with DNA of the plasmid construct ptsA58H (12), containing both a selectable marker, the hygromycin-resistance gene (21), and the sequence encoding the temperature-sensitive SV40 TAg tsA58 (19). Cells expressing tsA58 are transformed at the permissive temperature of 34°C but revert to the untransformed phenotype at the restrictive temperature of 39.6°C (12, 25–27). Because the

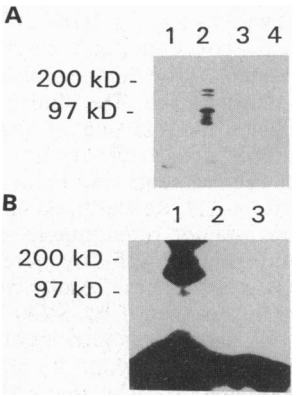

FIG. 1. R$^-$ cells do not express IGF-IR. (*A*) Autophosphorylation of the IGF-IR in W and R$^-$ mouse embryo cells. Procedure was the same as described for human IGF-IR (23, 25), except that, for mouse cells, we used an antibody to β subunit of mouse IGF-IR. Lanes: 1, lysate from wild-type embryo cells, no IGF-I added; 2, same as lane 1 but 15 min after IGF-I (20 ng/ml) addition; 3, lysate from R$^-$ cells, no IGF-I added; 4, same as for lane 3 after IGF-I. (*B*) Cross-linking of ^{125}I-labeled IGF-I to R$^-$ and wild-type mouse embryo cells. Cross-linking procedure was done as described. Lanes: 1, lysate from wild-type embryo cells; 2, lysate from wild-type embryo cells with unlabeled IGF-I at 500 ng/ml; 3, lysate from R$^-$ cells. The IGF-IR α subunit is 135 kDa in size.

selectable marker and TAg are expressed from the same plasmid, all hygromycin-resistant clones derived were also TAg positive. Thus, when selected W and R^- cells harboring ptsA58H were examined by immunofluorescence with an antibody against TAg, both cell types exhibited approximately the same level of intensity in staining (45 ± 0.9 and 43.7 ± 0.7 arbitrary densitometric units, respectively; Fig. 2). These transfected derivatives of parental W and R^- lines were designated (tsA)W and (tsA)R^- cells.

The four types of cells [W, R^-, (tsA)W, and (tsA)R^-] were plated and grown in DMEM/10% serum for 5 days, and then cell numbers were determined to assess saturation densities (Fig. 3). As expected from previous results (see above), the ratio of R^- to W cell numbers was 0.53. However, growth was differentially stimulated by SV40 TAg. The number of (tsA) W cells was 2.7-fold higher than that of W cells, whereas (tsA)R^- cells grew only 30% above saturation density of the R^- parent. More importantly, the (tsA)W cells were overtly transformed, as evidenced by the appearance of large foci, whereas the (tsA)R^- cells continued to be contact-inhibited. Identical results were obtained with several different TAg-positive clones derived from the parental cell lines.

To assess further the presence or absence of a transformed phenotype, we used soft-agar assays (9). Results of a typical experiment are shown in Table 2 (variability between different clones of the same cell type was negligible). As expected, (tsA)W cells formed colonies in soft agar in numbers that increased with the number of cells seeded, whereas only a single small colony of 12 cells appeared with the highest number of plated (tsA)R^- cells. The cells were maintained in 10% serum for >3 weeks, which is a more than adequate time period for establishment of colonies in soft agar, even at reduced growth rates. Therefore, the (tsA)R^- cells do not have the potential to form colonies in soft agar.

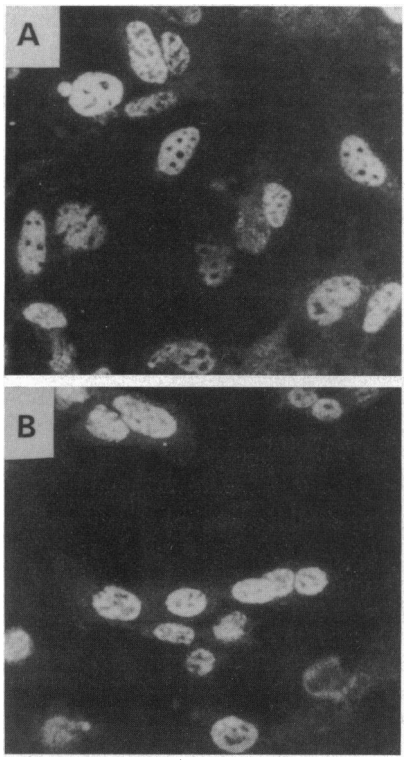

FIG. 2. Expression of SV40 TAg in (tsA)W and (tsA)R^- cells. Parental W and R^- cells were transfected with a plasmid containing a selectable marker and the tsA58 mutant of SV40 TAg. All selected clones were T positive, and 100% of the cells expressed TAg, as shown by immunostaining. (*A*) (tsA)W cells. (*B*) (tsA)R^- cells. (×470.)

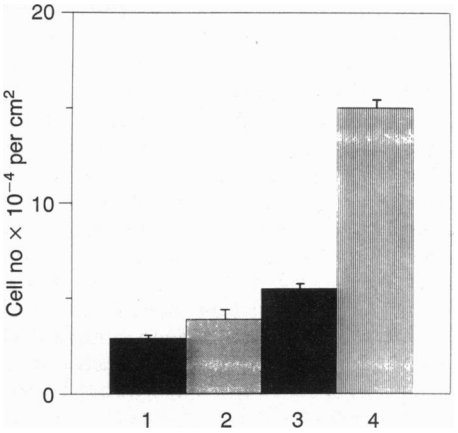

FIG. 3. Saturation densities of wild-type and mutant cells, either parental or transfected with SV40 TAg. Cells were seeded at the same concentration of 10 × 10^3 cells per cm^2 and grown in DMEM/10% fetal calf serum. The ordinate indicates the number of cells 5 days after seeding. Bars: 1, R^- cells; 2, R^- cells stably transfected with tsA58 expression plasmid [(tsA) R^- cells)]; 3, wild-type embryo cells, W cells; 4, W cells stably transfected with tsA58 TAg, (tsA)W cells. Small bars represent SDs of triplicate experiments.

To exclude the possibility that the temperature-sensitive TAg somehow differs from wild-type antigen, additional lines were derived from parental W and R^- cells by cotransfecting two plasmids expressing wild-type SV40 TAg and the hygromycin-resistance gene, respectively. After hygromycin selection, cells positive for TAg expression were expanded into clones that were assayed for colony formation in soft agar. Again, in contrast with controls, the derivatives of R^- cells could not form colonies (Table 2).

Although the described results were unequivocal and reproducible, it could still be argued that our observations were the indirect consequence of an irreversibly altered phenotype of mutant cells that underwent crisis. Thus, an additional experiment was done to show that the ability of SV40 TAg to transform fibroblasts depends directly on the presence of

Table 2. Growth in soft agar of mouse embryo cells expressing SV40 TAg

Cell type	Seeding density, no. × 10^{-3}	Colonies, no.
(tsA)W	1	8
	10	64
	100	350
(tsA)R^-	1	0
	10	0
	100	1
(wtT)W	10	58
	100	154
(wtT)R^-	10	0
	100	0
(tsA)R^+	10	16
	100	70

(tsA)W and (tsA)R^- are, respectively, W and R^- cells expressing tsA58 TAg. The same embryo cells expressing wild-type TAg are designated (wtT). (tsA)R^+ cells are (tsA)R^- cells expressing a stably transfected human IGF-IR cDNA. Parental cell lines W and R^- did not grow in soft agar (data not shown). Cells were seeded at the indicated densities, and colonies were counted after 25 days in culture. Numbers are the averages of duplicate counts of a single experiment. Several assays were done with reproducible results. Several clones of (tsA) and (wtT) cells were also tested with similar results. Although efficiency of colony formation varied slightly from experiment to experiment (even in experiments with high efficiency of colony formation), TAg-transfected R^- cells failed to produce colonies (except for one colony in a single experiment).

functional IGF-IR. This experiment was based on the observation that cells expressing IGF-IR constitutively can grow in serum-free medium supplemented only with IGF-I or with insulin at supraphysiological concentrations (23, 28). Thus, we transfected one of the (tsA)R$^-$ clones with a plasmid (Cvn-IGF-IR) expressing the full-length coding sequence of human Igf1r cDNA and also the neomycin-resistance gene, both under the control of the SV40 promoter (22). We selected clones directly in serum-free medium supplemented with insulin (20 μg/ml); under these conditions, only clones constitutively expressing the IGF-IR can grow (23), and, indeed, by autophosphorylation analysis these clones did express IGF-IR at levels comparable to those of BALB/c 3T3 cells (data not shown). These clones could now efficiently form colonies in soft agar (Table 2) without IGF-I addition. Therefore, the endogenously produced TAg, which was previously ineffective, realized its transforming potential once the cells acquired constitutively expressed human IGF-IR.

IGF-IR Requirement for Growth of Glioblastoma Cells in Soft Agar. Previous indirect data, showing that NIH 3T3 cells overexpressing IGF-IR grow in soft agar in the presence of the ligand (29) and that the tumorigenicity of the rat glioblastoma C6 cell line is abrogated by antisense Igf-1 RNA (17, 18), are consistent with our direct results obtained with mutant cells lacking IGF-IR. The data with C6 cells, considered together with ours, suggest that IGF-IR-mediated signaling of IGF-I is an indispensable component for operation of a transformation pathway. To show that this is, indeed, the case and that our observations are not a specific feature of embryonic mouse fibroblasts, we used an antisense RNA strategy with C6 cells and also with cells of an additional glioblastoma cell line, T98G (16), which grow well in 1% serum.

T98G glioblastoma cells were transfected with appropriate constructs to derive cell lines expressing either antisense or (control) sense human Igf1r RNA. Soft agar assays using these derivatives showed that, in comparison with control cells, the number of colonies formed by T98G cells expressing Igf1r antisense RNA was reduced >60-fold (Table 3). In an analogous experiment, we observed that C6 cell-colony formation in soft agar was reduced 2-fold in the presence of an antisense oligodeoxynucleotide-inhibiting Igf1r mRNA (Table 3). Interestingly, the growth of C6 cells in culture dishes was not reduced >10% in the presence of the same concentration of antisense oligodeoxynucleotides, whereas the growth rate of T98G cells expressing antisense RNA was 40% that of wild-type cells or cells expressing sense RNA. These observations are consistent with results from R$^-$ cells and suggest that the transformation phenotype is more sensitive to the abrogation or diminution of IGF-IR function than the inhibition of growth.

Table 3. Growth in soft agar of glioblastoma cell lines treated by antisense strategy to the IGF-IR

Cell type	Seeding density, no. × 10^{-3}	Colonies, no.
T98G-sense	3	305
T98G-antisense	3	5
C6	3	280
C6-sense	3	230
C6-antisense	3	115

Cells were seeded at the indicated densities, and colonies were counted after 14 days in culture. Both cell lines showed consistently higher efficiency of colony formation than TAg-transfected mouse embryo cells. T98G cells used are lines stably transfected with a heat shock promoter construct that transcribes either a sense or antisense transcript for the first 309 bp of IGF-IR. The T98G cells containing antisense construct grew at 40–50% the rate of lines containing sense construct. C6 cell line was treated with antisense oligonucleotide (80 μg/ml) to the IGF-IR known to reduce the IGF-IR level at the cell surface (23). The sequence used corresponds to the first 18 bp after the ATG initiation codon of IGF-IR cDNA. Addition of the antisense oligonucleotide had little effect on anchorage-dependent growth of these cells in 10% serum (data not shown).

DISCUSSION

Using a mutant cell background null for IGF-IR expression, we have provided direct evidence that the transforming potential of SV40 TAg depends on signaling via IGF-IR. This conclusion can be firmly reached by considering together two results: SV40 TAg was unable to transform (tsA) R$^-$ cells, but its transformation function was promptly re-established when these cells were transfected with a plasmid expressing human Igf1r cDNA.

In contrast to most other known oncoproteins, SV40 TAg is sufficient alone to transform a variety of cell types in culture and in transgenic animals (30, 31). In this regard, genetic analyses of SV40 TAg mutants have indicated that SV40 TAg possesses at least three transforming domains with variable, cell-type-dependent transformation potential (see refs. 9 and 32–34 and references therein). However, specificity cannot be necessarily assigned to these domains. Instead, endogenous factors differentially expressed in particular cells might be variably complementing different SV40 TAg mutants for the multiple SV40 TAg functions related to transformation. Furthermore, even when SV40 TAg is wild type, its transforming potential might be influenced by the cellular milieu. Thus, an important question pertains to the generality of our observations. Although limited at present, our data with mutant fibroblasts and also with glioblastoma cell lines examined by an antisense RNA strategy suggest that the significance of signaling via IGF-IR as a prerequisite of SV40 TAg transforming potential is not restricted to a unique cell type. Nevertheless, we do not expect that the IGF-IR system could operate in all cell types because adult hepatocytes and some cell lines from B-cell lymphomas, for example, do not possess IGF-I-binding sites (3, 35).

At present, speculations attempting to mechanistically explain the failure of SV40 TAg to transform fibroblasts lacking IGF-IR function are unwarranted, despite several interesting correlations that can be related to this phenomenon. For example, IGF-IR overexpression results in transformation (29); IGF-IR is constitutively phosphorylated in cells transformed by the *src* oncogene (36); IGF-I signaling via IGF-IR regulates the level of cdc2 mRNA (37), whereas cdc2 is apparently involved in the phosphorylation of both SV40 TAg (38) and the retinoblastoma gene product (39); SV40 TAg is a strong inducer of IGF-I and Igf1r mRNA (12, 13); and the retinoblastoma gene product stimulates transcription from one of the *Igf-2* gene promoters (40), whereas Wilms tumor suppressor protein (WT1) has suppressing activity both for this promoter (41) and the promoter of the *Igf1r* gene (42). However, this fragmented information does not yet cohere. It is important to recognize that complex networks of interactions are undoubtedly involved in the regulatory cascades resulting in transformation. Thus, perturbation of different pathways could lead to a transformed phenotype. For example, the E5 transforming protein of bovine papilloma virus, which binds to the PDGF receptor and activates it constitutively (43), cannot transform mammary epithelial cells lacking PDGF receptors (44). This phenomenon and other observations, including the case that we have studied, point to common elements between cell growth and cell transformation. Interestingly, despite this strong relationship, our results allow us to discriminate between transformation and mitogenesis.

Signaling via the IGF-IR is not a *sine qua non* requirement for the cell cycle to occur. In fact, our results with R$^-$ cells

grown in 10% serum indicate that a distinct mitogenic pathway exists, in which the IGF system does not participate because an unknown "growth factor," of extremely low serum concentration, can sustain proliferation. We suggest an "unknown" growth factor, because several known growth factors (including insulin, IGF-II, fibroblastic growth factor, transforming growth factor β, and others) were tested; none of them promoted growth of R^- cells (data not shown). However, even in 10% serum the cell cycle is retarded without IGF-IR (see Table 1). Thus, the IGF system is indispensable for maintaining the cell cycle at a normal rate. At the same time, the IGF-IR pathway, but not the alternative mitogenic process, is also involved in the SV40 TAg transformation network that is not affected by serum concentration. The implication is that growth and transformation can be separated at the molecular level.

Although assessment of the relationship between transformation and the processes related to cell-cycle rate is currently difficult, some interesting clues exist. Cultured fibroblasts that are competent to progress to S phase after exposure to growth factors, such as PDGF, pass through two control points in G_1, V and R (restriction). Cells are arrested in V stage, if not provided with essential amino acids, whereas after R stage they are committed to enter the S phase, regardless of environmental signals. Importantly, after V stage, no other growth factor except IGF-I is required for progression to R stage (45). Whether the responses elicited by the IGF-I/IGF-IR signaling events during this period and their orderly timing also relate to transformation and are relevant to known pathways remains to be seen. Involvement of the IGF system in growth and transformation opens the question of the potential participation of the IGF system in tumorigenesis. Despite a plethora of circumstantial evidence from a variety of tumors (46, 47), this possibility has yet to be examined critically in animal models. In view of our results, such investigation is now timely, especially because of the exciting prospect that interference with the IGF-IR autocrine loop could lead to clinical applications (17, 18).

This work was supported by Grants GM 33694 and CA 56309 to R.B., Grant AA-0123 to R.R., and by Grant HD28342 to A.E. from the National Institutes of Health.

1. Liu, J.-P., Baker, J., Perkins, A. S., Robertson, E. J. & Efstratiadis, A. (1993) *Cell* **75**, 59–72.
2. Lowe, W. L., Jr. (1991) in *Insulin-Like Growth Factors: Molecular and Cellular Aspects*, ed. LeRoith, D. (CRC, Boca Raton, FL), pp. 49–85.
3. Werner, H., Woloschak, M., Stannard, B., Shen-Orr, Z., Roberts, C. T., Jr., & LeRoith, D. (1991) in *Insulin-Like Growth Factors: Molecular and Cellular Aspects*, ed. LeRoith, D. (CRC, Boca Raton, FL), pp. 17–47.
4. Baserga, R. & Rubin, R. (1993) *Crit. Rev. Eukaryotic Gene Expression* **3**, 47–61.
5. Baserga, R. (1993) *Receptor* **2**, 261–266.
6. Topp, C. W., Lane, D. & Pollack, R. (1980) in *DNA Tumor Viruses*, ed. Tooze, J. (Cold Spring Harbor Lab. Press, Plainview, NY), pp. 205–296.
7. Ponten, J. (1971) *Spontaneous and Virus Induced Transformation in Cell Culture* (Springer, New York).
8. Chen, S. & Paucha, E. (1990) *J. Virol.* **64**, 3350–3357.
9. Thompson, D. L., Kalderon, D., Smith, A. E. & Tevethia, M. J. (1990) *Virology* **178**, 15–34.
10. Deppert, W., Kurth, M., Graessmann, M., Graessmann, A. & Knippschild, U. (1991) *Oncogene* **6**, 1931–1938.
11. Fanning, E. (1992) *J. Virol.* **66**, 1289–1293.
12. Porcu, P., Ferber, A., Pietrzkowski, Z., Roberts, C. T., Adamo, M., LeRoith, D. & Baserga, R. (1992) *Mol. Cell. Biol.* **12**, 5069–5077.
13. Ferber, A., Chang, C.-D., Sell, C., Ptasznik, A., Cristofalo, V. J., Hubbard, K., Ozer, H. L., Adamo, M., Roberts, C. T., Jr., LeRoith, D., Dumenil, G. & Baserga, R. (1993) *J. Biol. Chem.* **268**, 17883–17888.
14. Wharton, W. & Smyth, M. J. (1989) in *Cell Growth and Division*, ed. Baserga, R. (IRL, Oxford), pp. 139–153.
15. Todaro, G. J. & Green, H. (1963) *J. Cell Biol.* **17**, 299–312.
16. Stein, G. H. (1979) *J. Cell. Physiol.* **99**, 43–54.
17. Trojan, J., Blossey, B. K., Johnson, T. R., Rudin, S. D., Tykocinski, M., Ilan, J. & Ilan, J. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 4874–4878.
18. Trojan, J., Johnson, T. R., Rudin, S. D., Ilan, J., Tykocinski, M. L. & Ilan, J. (1993) *Science* **259**, 94–97.
19. Tegtmeyer, P. (1975) *J. Virol.* **15**, 613–618.
20. Floros, J., Jonak, G., Galanti, N. & Baserga, R. (1981) *Exp. Cell Res.* **132**, 215–223.
21. Gritz, L. & Davies, J. (1983) *Gene* **25**, 179–188.
22. Ullrich, A., Gray, A., Tam, A. W., Yang-Feng, T., Tsubokawa, M., Collins, C., Henzel, W., Le Bon, T., Katchuria, S., Chen, E., Jacobs, S., Francke, D., Ramachandran, J. & Fujita-Yamaguchi, Y. (1986) *EMBO J.* **5**, 2503–2512.
23. Pietrzkowski, Z., Lammers, R., Carpenter, G., Soderquist, A. M., Limardo, M., Phillips, P. D., Ullrich, A. & Baserga, R. (1992) *Cell Growth Differ.* **3**, 199–205.
24. Yamori, T., Iizuka, Y., Takayama, Y., Nishiya, S., Iwashita, S., Yamazaki, A., Takatori, T. & Tsuruo, T. (1991) *Cancer Res.* **51**, 5859–5865.
25. Jat, P. S. & Sharp, P. A. (1989) *Mol. Cell. Biol.* **9**, 1672–1681.
26. Radna, R. L., Caton, Y., Jha, K. K., Kaplan, P., Li, G., Traganos, F. & Ozer, H. L. (1989) *Mol. Cell. Biol.* **9**, 3093–3096.
27. Resnick-Silverman, L., Peng, Z., Li, G., Jha, K. K. & Ozer, H. L. (1991) *J. Virol.* **65**, 2845–2852.
28. McCubrey, J. A., Stillman, L. S., Mayhew, M. W., Algate, P. A., Dellow, R. A. & Kaleko, M. (1991) *Blood* **78**, 921–929.
29. Kaleko, M., Rutter, W. G. & Miller, A. D. (1990) *Mol. Cell. Biol.* **10**, 464–473.
30. Livingston, D. M. & Bradley, M. K. (1987) *Mol. Biol. Med.* **4**, 63–80.
31. Adams, J. M. & Cory, S. (1991) *Science* **254**, 1161–1167.
32. Kierstead, T. D. & Tevethia, M. J. (1993) *J. Virol.* **67**, 1817–1829.
33. Tedesco, D., Fischer-Fantuzzi, L. & Vecso, C. (1993) *Oncogene* **8**, 549–557.
34. Symonds, H. S., McCarthy, S. A., Chen, J., Pipas, J. M. & Van Dyke, T. (1993) *Mol. Cell. Biol.* **13**, 3255–3265.
35. Lee, P. D. K., Rosenfeld, R. G., Hintz, R. L. & Smith, S. D. (1986) *J. Clin. Endocr. Metab.* **62**, 28–35.
36. Kozma, L. M. & Weber, M. J. (1990) *Mol. Cell. Biol.* **10**, 3626–3634.
37. Surmacz, E., Nugent, P., Pietrzkowski, Z. & Baserga, R. (1992) *Exp. Cell Res.* **199**, 275–278.
38. Fanning, E. & Knippers, R. (1992) *Annu. Rev. Biochem.* **61**, 55–85.
39. Hollingsworth, R. E., Chen, P.-I. & Lee, W.-I. (1993) *Curr. Opin. Cell Biol.* **5**, 194–200.
40. Kim, S. J., Onwuta, U. S., Lee, Y. I., Li, R., Botchan, M. R. & Robbins, F. D. (1992) *Mol. Cell. Biol.* **12**, 2455–2463.
41. Drummond, I. A., Madden, S. L., Rohwer-Nutter, P., Bell, G. I., Sukhatme, V. P. & Rauscher, F. L. (1992) *Science* **257**, 674–678.
42. Werner, H., Re, G. G., Drummond, I. A., Sukhatme, V. P., Rauscher, F. J., III, Sens, D. A., Garvin, A. J., LeRoith, D. & Roberts, C. T., Jr. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 5828–5832.
43. Petti, L., Nilson, L. A. & DiMaio, D. (1991) *EMBO J.* **10**, 845–855.
44. Nilson, L. A. & DiMaio, D. (1993) *Mol. Cell. Biol.* **13**, 4137–4145.
45. Pardee, A. B. (1989) *Science* **246**, 603–608.
46. Cullen, K. J., Yee, D. & Rosen, N. (1991) *Cancer Invest.* **9**, 443–454.
47. Macaulay, V. M. (1992) *Br. J. Cancer* **65**, 311–320.