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The Growth-Stimulatory Effect of Simian Virus 40 T Antigen Requires the Interaction of Insulinlike Growth Factor 1 with Its Receptor

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We have used a plasmid expressing a temperature-sensitive (*ts*) mutant of simian virus 40 (SV40) T antigen, stably transfected into 3T3 cells, to study the role of insulinlike growth factor 1 (IGF-1) and its receptor in T-antigen-mediated growth. While 3T3 cells do not grow in serum-free medium, in 1% serum, or with the sole addition of either platelet-derived growth factor (PDGF) or IGF-1, cells expressing the *ts*A T antigen (BALB 58 cells) grow at 34°C in either PDGF or 1% serum but not in IGF-1. At the restrictive temperature (39.6°C), these cells can only grow in 10% serum. We show that BALB 58 cells, at 34°C, have a markedly increased expression of IGF-1 and IGF-1 mRNA and that their growth in 1% serum (at 34°C) is inhibited by an antisense oligodeoxynucleotide to the IGF-1 receptor RNA. When this *ts*A plasmid is stably transfected into cells constitutively overexpressing the human IGF-1 receptor cDNA, the resulting cell lines show a constitutively phosphorylated IGF-1 receptor and grow in serum-free medium at 34°C (but not at 39.6°C). A functional SV40 T antigen also increases the expression of a plasmid in which the reporter luciferase gene is under the control of a rat IGF-1 promoter. We conclude (i) that the SV40 T antigen induces the expression of IGF-1 and IGF-1 mRNA, at least in part by a transcriptional mechanism, thus altering the growth factors requirements, and (ii) that, in BALB/c3T3 cells, the SV40 T antigen necessitates a functional IGF-1 receptor for its growth-stimulating effect in low serum (or PDGF).

The growth of cells in culture is regulated by growth factors (both stimulatory and inhibitory) in the environment. BALB/c3T3 cells are exquisitely growth regulated (44) and require at least two growth factors for optimal growth, usually platelet-derived growth factor (PDGF) and insulin-like growth factor 1 (IGF-1) (17, 27, 47). The requirement for PDGF can be abrogated by overexpression of c-*myc* (3, 23). Recently, we reported that the proto-oncogene c-*myb* abrogated the requirement for IGF-1, but not for PDGF, by increasing the expression of both IGF-1 and the IGF-1 receptor RNA (39, 52). The simian virus 40 (SV40) T antigen transforms cells in culture and decreases the requirements for growth factors; typically, SV40-transformed cells can grow in 1% serum (45, and for a review, see reference 51). Because 1% serum is often considered to be the equivalent of PDGF only, we have asked whether the SV40 T antigen, like c-*myb*, may also induce the expression of IGF-1 and require, to stimulate growth, the activation of the IGF-1 receptor by its ligand. For this purpose, we have stably transfected into BALB/c3T3 cells (and derivative cell lines) a plasmid, pts58, expressing a temperature-sensitive (*ts*) mutant of the SV40 T antigen (49). Cells transformed with the *ts* T antigen behave like transformed cells at 34°C but revert to the untransformed phenotype at the restrictive temperature of 39 to 39.6°C (22, 37). Our results show that in BALB/c3T3 cells the SV40 T antigen increases the expression of IGF-1 and IGF-1 mRNA and requires a functional IGF-1 receptor to stimulate growth in low serum.

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MATERIALS AND METHODS

Plasmids. pts58 contains the *ts*A58 T antigen coding gene (49), cloned in pBR322 (gift of W. E. Mercer). pLHL4 (19) carries the hygromycin resistance gene. ptsA58H was constructed in our laboratory; it contains the *ts*A58 SV40 large T antigen, the hygromycin resistance hph cDNA under the control of the herpes simplex virus type 1 thymidine kinase promoter and with the herpes simplex virus type 1 polyadenylation signal, and the β-lactamase gene and origin of replication of pBR322. Another plasmid discussed in this paper is Cvn-IGF-1 receptor, which has the full-length coding sequence of human IGF-1 receptor cDNA plus the neomycin resistance gene, both under the control of the SV40 promoter (26, 53).

Construction of rat IGF-1 promoter-luciferase plasmids. The promoterless plasmid containing the luciferase cDNA, p0Luc, was kindly provided by Alan Brasier, Harvard University, Cambridge, Mass. Two exon 1 DNA fragments were derived from a rat IGF-1 genomic clone (7) and are presented schematically in Fig. 1. The first is a 324-bp *Sau*3A-*Fsp*I fragment containing the three exon 1 transcription start sites described by Adamo et al. (1) and includes 134 bp upstream of start site 1,172 bp upstream of start site 2 and ~270 bp upstream of start site 3. This fragment extends to ~56 bp downstream of start site 3. This fragment was directionally subcloned into the *Bam*HI and blunt-ended *Hin*dIII sites of p0Luc generating the IGF luc plasmid. The second fragment, also derived from a rat IGF-1 genomic clone, was an ~1.48-kb *Sma*I-*Bgl*II fragment. This fragment includes all exon 1 transcription start sites (2) and contained 1,121 bp upstream of start site 1, 1,159 bp upstream of start site 2, 1,256 bp upstream of start site 3, and 1,468 bp

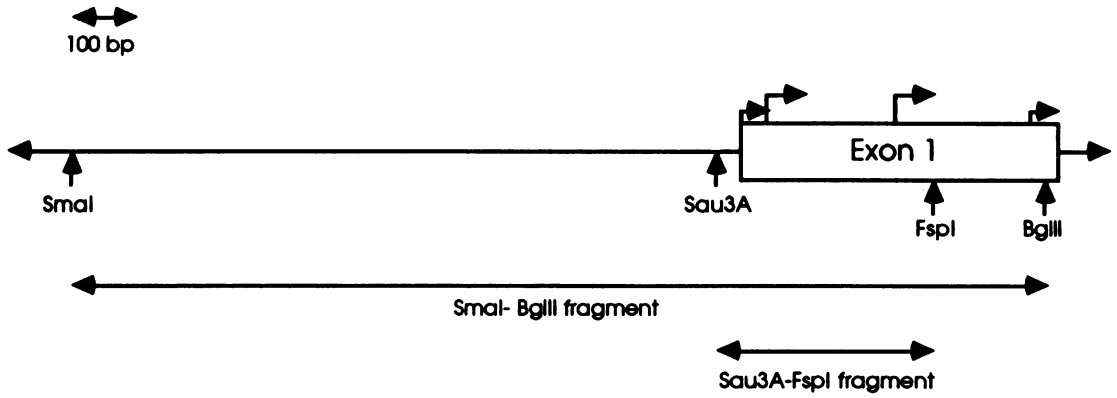

FIG. 1. Schematic representation of the rat IGF-1 promoter and exon 1, with the restriction sites relevant to this paper (see text).

upstream of start site 4. This fragment extends ~37 bp past the AUG initiating preproIGF-1 translation and ends 25 bp upstream of the 3′ end of exon 1. It was initially subcloned (after blunting of the *Bgl*II 5′ overhang) into the *Sma*I site of pGEM4Z. After the orientation was determined by sequence analysis, the fragment was removed from the vector by digestion with *Bam*HI (to cleave just downstream of the 3′ end of the fragment), by blunt-ending of the *Bam*HI 5′ overhang, and then by digestion with *Xma*I (to cleave the 5′ *Sma*I site). This fragment was then directionally subcloned in the *Xma*I and blunt-ended *Hin*dIII sites of p0Luc, generating the IGF luc2 plasmid. All plasmids were purified with reagents from Quiagen (Los Angeles, Calif.).

Cell lines. 3T3 are BALB/c3T3 cells that have been grown for several years in our laboratory. They are grown in 10% serum (5% calf serum–5% fetal calf serum); 1% serum is fetal calf serum. BALB A58 cells were originated by cotransfection of plasmids pts58 and pLH4 and selection in hygromycin B (300 μg/ml). Other clones were generated by transfection with the pts58H plasmid, which includes the selectable marker (see above). After selection, all clones tested (several) were found to be 100% T positive by immunofluorescence (not shown).

p6 cells (35) are 3T3 cells constitutively overexpressing the human IGF-1 receptor cDNA (plasmid Cvn-IGF-1 R). They do not grow in serum-free medium (SFM), but they grow with the sole addition of IGF-1 (10 ng/ml or more). p6A cells were generated by transfecting p6 cells with plasmid ptsA58H and by selection with both neomycin (200 μg/ml) and hygromycin (300 μg/ml). luc A cells are 3T3 cells transfected with plasmid IGF luc, and the *neo* resistance gene. These cells were subsequently transfected with plasmid ptsA58H. Dual selection was again used, to generate a cell line we called luc A58.

Growth in SFM has been described in detail by Pietrzkowski et al. (35). Briefly, we do not use transferrin, but we supplement the medium with ferrous sulfate. For growth curves, cells were plated in 35-mm dishes at a concentration of 10^4 cells per dish, unless otherwise noted. PDGF (3 ng/ml) was from GIBCO, and IGF-1 (10 ng/ml) was from UBI.

RNA extraction. RNA was isolated by standard methods (9), but for reverse transcriptase (RT)-polymerase chain reaction (PCR), it was further centrifuged in a CsCl gradient, as previously described (29). Blots and labeling of probe were carried out by standard methods (16, 50).

RT-PCR. We followed the method of Rappolee et al. (38) as modified in our laboratory (29). The amplimers for mouse IGF-1 RNA were 5′ ATG TCG TCT TCA CAC CTC 3′ and 3′ AGG CTT CAG TGG GGC ACA 5′ and were based on the published mouse liver IGF-1 sequence (5). As probe, we used an oligonucleotide sequence corresponding to codons 105 to 123 (5). The luciferase amplimers, all based on the published cDNA sequence (14), were 5′ TAG AGG ATG GAA CCG CTG GA 3′ and 3′ CGC AAC TGC AAC TCC GAT AA 5′. The sequence GAA CAT CAC GTA CGC GGA AT was used as the probe. All oligodeoxynucleotides were synthesized on an Applied Biosystem, Inc., model 391 EP DNA synthesizer by using β-cyanoethyl phosphoromidite chemistry.

We use several controls in all our RT-PCR reactions: (i) samples that give a signal in the absence of RT (DNA contamination) are eliminated, (ii) we run different numbers of cycles (usually from 10 to 30), and (iii) we also amplify an RNA which is expressed at constant levels under different growth conditions. We often use the 3A10 insert (28), but in the present experiments we have also used β-actin, which gave constant signals in 1% serum at both 34 and 39.6°C. (iv) The experiments are always repeated several times with different RNA preparations. Amplimers for beta actin were 5′ TGG GAA TGG GTC AGA AGG ACT 3′ and 3′ TGG GAT TCC GGT TGG CAC TTT. The probe for hybridization was: 5′ AGA GGT ATC CTG ACC CTG AAG. All of them were based on GenBank sequences and are valid for both human and mouse β-actin.

Antisense oligodeoxynucleotides. These were synthesized as phosphorothioates. The antisense to IGF-1 receptor RNA was 5′ TCC TCC GGA GCC AGA CTT 3′, corresponding to codons 21 to 26 of the signal sequence preceding the proreceptor sequence of the IGF-1 receptor (53). The mismatched antisense was 5′ GCA CCG GGA AGT TGT GTC AA 3′. The oligomers were added at a concentration of 40 μg/ml.

Transient expression assay. For transient expression assays, the IGF luc plasmid (8 μg per dish) was transfected into BALB 58 cells. The single pool of transfected cells was then divided into two parts: one half was incubated at 34°C and the second half was incubated at 39.6°C. The amount of luciferase RNA was determined by RT-PCR at 48 h after transfection. Exactly the same experiment was repeated with BALB/c3T3 cells. Both the IGF luc and the IGF luc 2 plasmids were tested in transient assays.

IGF-1 radioimmunoassay. Conditioned medium containing 0.1% bovine serum albumin (BSA) and 1 mM ferric sulfite was collected at various times after the cells were transferred to SFM. To remove IGF-1 binding proteins, 0.1 ml of conditioned medium was mixed with 900 ml of 1 M acetic acid and 5% BSA and loaded onto SepPak C18 columns

(Waters, Milford, Mass.). Before loading, the column was washed with 10 ml of methanol and then by 10 ml of H_2O.

After loading, the column was washed with 10 ml of 4% acetic acid, and IGF-1 was eluted in 1 ml of 50% acetonitrile and 4% acetic acid. After lyophilization, the sample was resuspended directly in 100 ml of radioimmunoassay buffer. The assay was performed according to the instructions of the kit's manufacturer (Amersham, Arlington Heights, Ill.) with a rabbit IGF-1 anti-serum and a second antibody bound to magnetic beads (Amersham).

Phosphorylation of IGF-1 receptor. Cells were stimulated with the ligand at 37°C for 5 min. The cells were placed on ice and rinsed with cold Hanks buffered saline. Cells were lysed with lysis buffer (50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.5], 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 100 mM NaF, 0.2 mM sodium orthovanadate, 10 mM sodium PP_i) containing 10 mM phenylmethylsulfonyl fluoride and 0.1 mg of aprotinin per ml. After a 3-min lysis at 4°C, the lysate was centrifuged for 2 min at 4°C to remove nuclei. The cleared lysate was transferred to a fresh tube and 1 volume of HNTG (20 mM HEPES [pH 7.5], 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, 0.2 mM sodium orthovanadate, 10 mM NaF) was added. One microgram of monoclonal antibody to the IGF-1 receptor, αIR3 (Oncogene Sciences, Uniondale, N.Y.) was added. Anti-mouse immunoglobulin G-agarose conjugate (100 μg) (Sigma Immunochemicals, St. Louis, Mo.) was then added to immunoprecipitate the antibody-receptor complex. Antibody-antigen complexes were allowed to form for 2 h at 4°C and were then centrifuged at 4°C for 5 min. The complex was washed three times with HNTG, and 20 μl of Laemmli buffer (20% glycerol, 3% sodium dodecyl sulfate, 3% β-mercaptoethanol, 10 mM EDTA, 0.05% bromophenol blue) was added. Samples were boiled for 5 min, and proteins were separated on a 7% polyacrylamide gel. Proteins were then electroblotted onto a nitrocellulose filter. Phosphorylated proteins were detected by Western immunoblot analysis by using standard techniques with an anti-phosphotyrosine antibody (UBI, Saranac Lake, N.Y.). Detection of bound phosphotyrosine antibodies was carried out by using the ECL detection system from Amersham.

RESULTS

Transformed clones were generated by cotransfecting the pts58 (carrying the SV40 T antigen gene of mutant *ts*A58) and pLH4 (hygromycin resistance gene) plasmids. Clones were selected in hygromycin at 37°C in 10% serum. Several T-positive clones were investigated for their growth properties; since all of them behaved similarly (see below), we eventually selected two clones (clones A and L) for more detailed studies. Several reports from the literature (13, 22, 37, 41) indicate that a number of functions of the *ts*A58 T antigen are abrogated when the cells are incubated at the restrictive temperature (see Discussion). All clones tested had 100% of the cells T positive by immunofluorescence (not shown).

Growth factor requirements of 3T3 cells transformed with an SV40 *ts*A mutant. In these experiments, cells were plated at a concentration of 10^4 cells per dish. The number of cells was determined daily for 6 days. Figure 2 shows a typical result giving the number of cells per dish on day 6. The parent cell line, BALB/c3T3, as expected, did not grow in 1% serum but did grow in 10% serum, both at 34 and 39.6°C. Two SV40-transformed clones are shown in Fig. 2, but

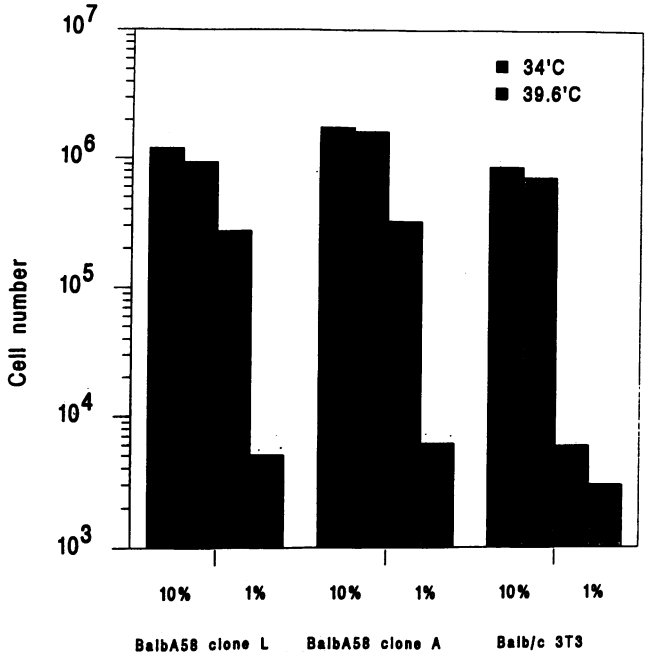

FIG. 2. Growth of 3T3 and BALB A58 Cells. 3T3 and two clones of 3T3 cells stably transfected with the SV40 *ts*A58 mutant (BALB A58) were plated at a concentration of 10^4 cells per dish. The cells were grown in either 10 or 1% serum at either 34 or 39.6°C. The number of cells per dish (ordinate) was determined on day 6 after plating.

several other clones gave the same results. The BALB A58 clones (expressing a *ts* T antigen) grow in 1% serum at 34 but not at 39.6°C. A serum concentration of 1% is often said to be the equivalent of PDGF. As reported in the literature (17, 27, 44, 52), 3T3 cells can grow in SFM with the addition of PDGF and IGF-1. We repeated the experiments of Fig. 2, but in SFM, with the addition of either PDGF or IGF-1. The results of a representative experiment are shown in Fig. 3, where growth in 1% serum is given for comparison. Again, 3T3 cells do not grow in PDGF or IGF-1 alone or 1% serum; at 34°C, both clone A and clone L grow in SFM plus PDGF but grow very little in SFM plus IGF-1. At 39.6°C, cells actually decrease in number (not shown). Also not shown are data that neither 3T3 nor the A58 clones can grow in SFM alone (see below).

The results, thus far, indicate that an active SV40 T antigen can abrogate the requirement for IGF-1 but can do so only very little for PDGF. Travali et al. (52) had previously shown that a constitutively overexpressed c-*myb* can also abrogate the IGF-1 requirement for 3T3 cells. However, it was found that, under these conditions, the *myb* product increased the expression of both IGF-1 and IGF-1 receptor RNAs (39, 52). We asked whether the SV40 T antigen could also act in the same way.

SV40-transformed cells have increased expression of IGF-1 and IGF-1 mRNA. We determined the levels of IGF-1 mRNA by RT-PCR with the amplimers and probes described in Methods and Materials. BALB 58 cells were maintained in 1% serum, at either 34 or 39.6°C. BALB/c3T3 cells, at 37°C, were either quiescent or stimulated with 10% serum. One such experiment is shown in Fig. 4. At 34°C, clones A and L (SV40 transformed) gave a clear signal for IGF-1 mRNA (panel A), detectable even after 20 cycles of amplification.

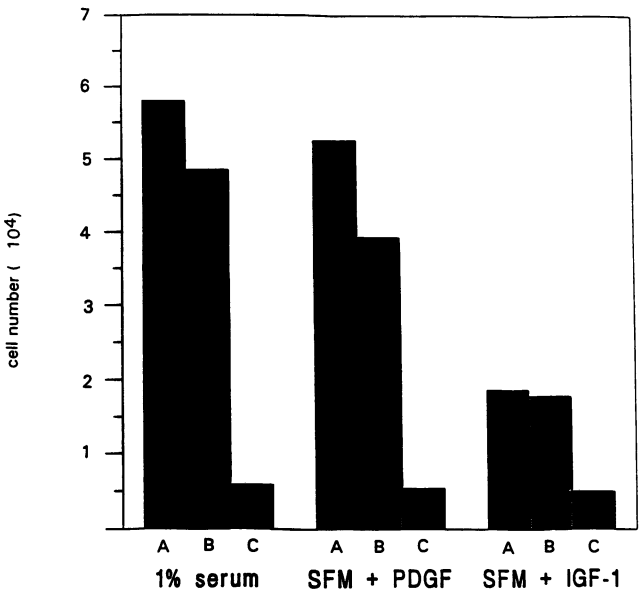

FIG. 3. Growth of BALB A58 cells in SFM plus individual growth factors. Cells were plated as described in Methods and Materials; after 24 h they were incubated in SFM at 34°C with the indicated additions. The number of cells per dish (ordinate) was determined after an additional 48 h. A, BALB A58, clone A; B, BALB A58, clone L; C, BALB/c3T3.

At the restrictive temperature, no IGF-1 mRNA is detectable, even after 30 cycles of amplification under the conditions used. A weak signal is detectable in 3T3 cells after serum stimulation; mouse liver mRNA (the mouse liver is a major site of production of IGF-1 [55]) gave the strongest signal. The results are not explainable by a trivial temperature effect; in the same cells, the levels of β-actin mRNA are essentially the same, in the three cell lines and at either temperature (Fig. 4B). In fact, incubation of 3T3 cells at 39.6°C in 10% serum causes a small increase in IGF-1 mRNA (not shown). This induction of IGF-1 mRNA by temperature

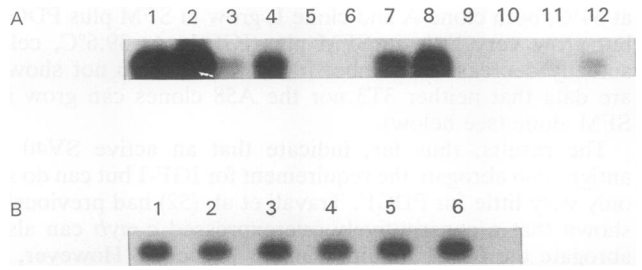

FIG. 4. Expression of IGF-1 mRNA in 3T3 and BALB A58 Cells. (A) IGF-1 mRNA; (B) is β-actin. 3T3 cells were either quiescent (lane 11) or serum stimulated (lane 12). Clones A and L of BALB A58 cells were in 1% serum. (A) Lanes: 1 and 2, mouse liver mRNA, 20 and 30 cycles of amplification, respectively; 3 and 4, clone A at 34°C, 20 cycles and 30 cycles, respectively; 5 and 6, clone A at 39.6°C, 20 and 30 cycles, respectively; 7 and 8, clone L at 34°C, 20 and 30 cycles, respectively; 9 and 10, clone L at 39.6°C, 20 and 30 cycles, respectively; 11, quiescent; and 12, serum-stimulated 3T3 cells, 30 cycles. (B) β-actin RNA levels in the same cells as in panel A (only 15 cycles of amplification). Lanes: 1 and 2, clone A at 34 or 39.6°C; 3 and 4, clone L at 34 or 39.6°C; 5 and 6, 3T3 cells. Amplimers, probe, and RT-PCR protocol are given in Methods and Materials.

TABLE 1. Concentration of IGF-1 in the medium conditioned by BALB A58 cells[a]

Temperature	Treatment in SFM (h)	IGF-1 (ng/ml/ 10^6 cells)
34°C	1.5	3.0
	4.0	3.8
	8.0	5.2
	16.0	8.0
39.6°C	1.5	0.4
	4.0	0.4
	8.0	0.8
	16.0	0.6

[a] BALB A58 cells (clone A) were grown as usual in 1% serum. At zero time, growth medium was replaced by SFM, and the concentration of IGF-1 was determined, at the time indicated, by the radioimmunoassay described in Methods and Materials.

in 10% serum is presently being investigated but will not be discussed further in this paper. In 1% serum at 34°C, T antigen increases the expression of IGF-1 RNA by many fold. We also asked whether the T antigen would affect the levels of IGF-1 receptor RNA. We found no difference in IGF-1 receptor RNA levels in BALB 58 cells between 34 and 39.6°C (data not shown).

Table 1 shows that IGF-1 itself is secreted in the medium of BALB 58 cells (clone A) at 34°C. The amount of IGF-1 was determined by radioimmunoassay (see Methods and Materials) in cells incubated in SFM for the times indicated. The concentration of IGF-1 increases progressively with time of incubation in SFM at 34°C. With clone L, no IGF-1 was detectable in the medium at 39.6°C; at 34°C, the estimated amount at 16 h was 3 ng/ml.

The IGF-1 receptor is activated in SV40-transformed cells. After stimulation with IGF-1, the IGF-1 receptor is rapidly autophosphorylated (26). The autophosphorylation of the receptor can be detected by immunoprecipitation with an antibody to the IGF-1 receptor, blotting, and staining with a phosphotyrosine antibody (26). We reasoned that if SV40-transformed cells secrete IGF-1, the receptor should be phosphorylated even in SFM. For this purpose, we generated a new cell line as follows: recently, Pietrzkowski et al. (35) reported that 3T3 cells constitutively overexpressing the IGF-1 receptor, p6 cells, grow in SFM with the sole addition of IGF-1 (or insulin at supraphysiological concentrations). Under these conditions, the cells grow without activation of either the PDGF or epidermal growth factor (EGF) receptors. We transfected plasmid ptsA58H (containing also the hygromycin resistance gene) in p6 cells and selected clones growing in both neomycin and hygromycin. We generated these new clones for two reasons: (i) in p6 cells, the IGF-1 receptor is overexpressed (35), which makes easier the detection of its autophosphorylation; and (ii) at the same time, if SV40-transformed clones secrete sufficient amounts of IGF-1 for growth, and since p6 cells grow in IGF-1 only, clones expressing both T antigen and IGF-1 receptor should grow in SFM at 34 but not at 39.6°C. This latter prediction was confirmed, as shown in Fig. 5. p6A cells (as we called cells expressing both the *ts*A T antigen and the human IGF-1 receptor) grow in SFM at 34°C, while clone L requires PDGF (Fig. 3) and p6 cells require IGF-1 (35).

Figure 6 shows that in p6A cells growing in SFM at 34°C, the IGF-1 receptor is phosphorylated. Lane 1 is the control, p6 cells 10 min after addition of IGF-1. The other lanes are from pA6 cells in SFM without addition of exogenous

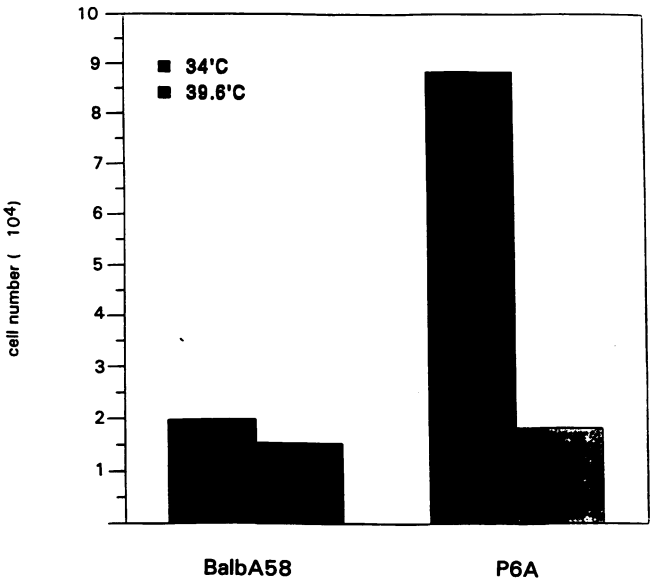

FIG. 5. Growth of p6A cells in SFM. p6 cells are 3T3-derived cells that constitutively overexpress the human IGF-1 receptor and grow in IGF-1 alone (35). They were stably transfected with the ptsA58H plasmid (which incorporates a selectable marker), and the resultant clones were found to express both T antigen and human IGF-1 receptor (p6A cells). These cells were plated as described in Methods and Materials and then incubated in SFM at either 34 or 39.6°C, and the number of cells was determined on day 5 (ordinate).

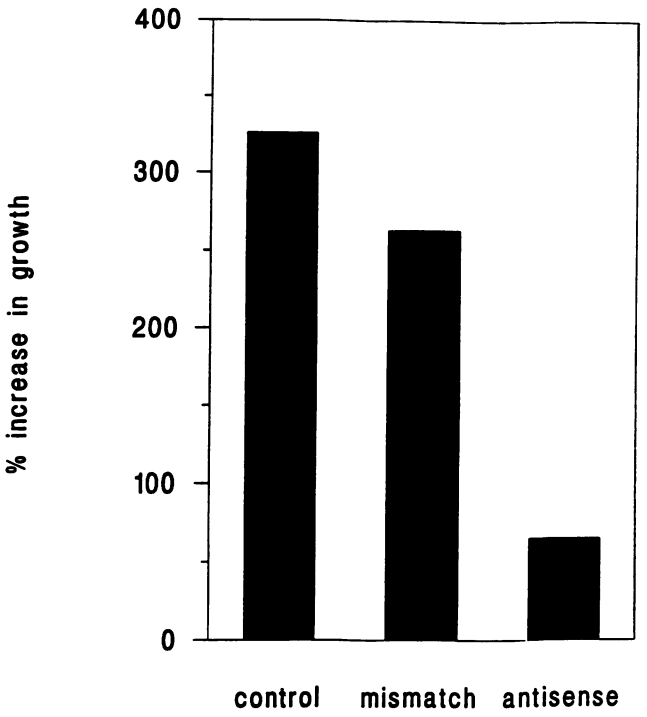

FIG. 7. Effects of an antisense oligodeoxynucleotide to IGF-1 receptor RNA on the growth of BALB 58 cells clone L. Cells, after plating, were incubated with an antisense (or a mismatched) oligodeoxynucleotide (phosphorothioate) at a concentration of 40 μg/ml. The cells were then grown in 1% serum, and the number of cells (ordinate) was determined after 72 h.

growth factors, at 39.6°C (lane 2) and at 34°C (lane 3). The IGF-1 receptor is constitutively autophosphorylated in pA6 cells growing at 34°C but not in cells incubated at 39.6°C.

An antisense oligodeoxynucleotide to IGF-1 R RNA inhibits the growth of SV40-transformed cells. In a previous paper, we had shown that an antisense oligodeoxynucleotide to IGF-1 R RNA inhibited the IGF-1-mediated growth of p6 cells (35). This antisense oligomer decreases the number of IGF-1 binding sites and the amount of autophosphorylated IGF-1 receptor (36). Figure 7 shows the effect of this antisense oligodeoxynucleotide to the IGF-1 receptor RNA on the growth of clone L cells in 1% serum at 34°C; there is an 85% inhibition of growth (versus control, untreated cells), while a mismatched oligodeoxynucleotide gave only a 15% inhibition.

Mechanism of T-antigen-mediated increase in IGF-1 expression. For this purpose, we generated another cell line (al-

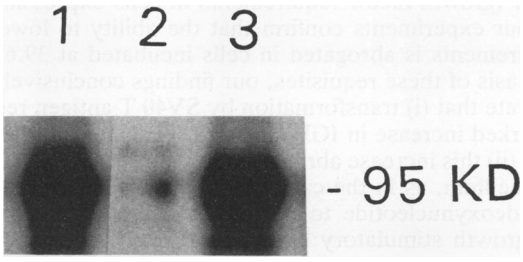

FIG. 6. Autophosphorylation of the IGF-1 receptor in p6A cells. The cells were grown in SFM at either 34 or 39.6°C. The autophosphorylation of the IGF-1 receptor was carried out as described in detail in Methods and Materials. Lanes: 1, p6 receptor in SFM 10 min after IGF-1; 2, p6A cells at 39.6°C in SFM; 3, p6A cells at 34°C in SFM (no IGF-1 was added to cells from lanes 2 and 3).

ways from 3T3 cells) coexpressing the *ts*A T antigen mutant and the luciferase reporter cDNA under the control of a rat IGF-1 promoter fragment (1). By using the amplimers for luciferase RNA described in Methods and Materials, no signal could be detected in untransfected 3T3 cells, even after 40 cycles of amplification (not shown). luc A cells (luciferase only, under the control of the rat *Sau*3A IGF-1 promoter) gave a clear signal in 10% serum, after 15 to 20 cycles of amplification. The intensity of the signal was the same at 34 and 39.6°C (Fig. 8, lanes 1 and 2 and 3 and 4). In luc A58 cells (expressing both *ts* A58 and the IGF-1 luc plasmids), luciferase mRNA levels were increased when the cells were incubated at 34°C over incubation at 39.6°C (Fig. 8, lanes 5 and 6 and 7 and 8). Note that, at 39.6°C, the signal at 30 cycles of amplification (lane 8) has the same intensity as at 34°C after only 20 cycles of amplification (lane 5). The endogenous IGF-1 mRNA, however, almost undetectable in luc A cells (no T antigen), is markedly overexpressed in luc A58 cells at 34°C (Fig. 8B, lanes 5 and 6). At 39.6°C (lanes 7 and 8), endogenous IGF-1 mRNA is barely detectable, even in luc A58 cells, under these conditions. This experiment was carried out in 1% serum. RT-PCR controls (not shown) indicated similar amounts of RNA in each reaction. As an additional control, we stimulated luc A and luc A58 cells with 10% serum: luciferase expression was markedly increased in all cases, indicating that this promoter is growth factor responsive (not shown).

In luc A58 cells, the difference in luciferase RNA levels at 34 versus 39.6°C (Fig. 8A) is not as marked as in endogenous IGF-1 mRNA levels (Fig. 8B). In addition, we do not know if the stably integrated plasmid IGF luc is under the influence

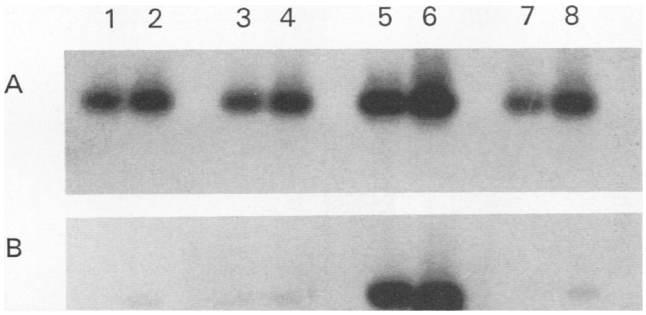

FIG. 8. Effects of SV40 T antigen on the IGF-1 promoter-directed transcription of the luciferase gene. Two 3T3 derivative cell lines were generated as follows: luc A was stably transfected with the expression plasmid IGF luc, in which a short IGF-1 promoter (Fig. 1) drives the luciferase gene. luc A58 cells are luc A cells stably transfected with ptsA58H plasmid, expressing the *ts* SV40 T antigen. Lanes 1 to 4, RNA from luc A cells; lanes 5 to 8, RNA from luc A58 cells. Each pair of number represents 20 and 30 cycles of RT-PCR. (A) RT-PCR of luciferase RNA; (B) RT-PCR of the endogenous IGF-1 mRNA. Amplimers and probes are given in Methods and Materials. Lanes: 1 and 2, luc A cells at 34°C; 3 and 4, luc A cells at 39.6°C; 5 and 6, luc A58 cells at 34°C; 7 and 8, luc A58 cells at 39.6°C.

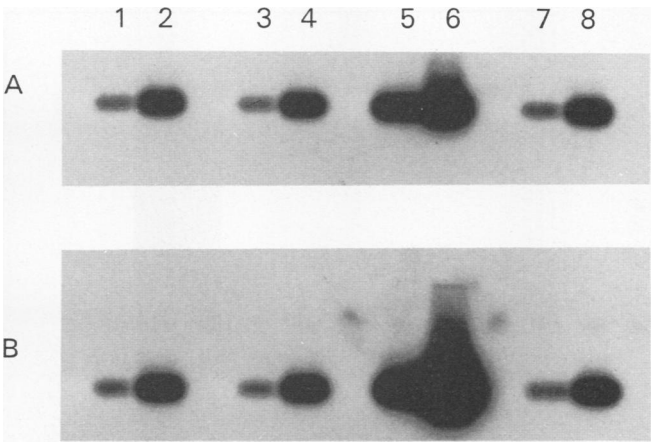

FIG. 9. Luciferase RNA levels in 3T3 and BALB A58 cells transiently transfected with the IGF luc plasmids. The cells were transfected with the designated expression plasmid, and each transfection pool was divided into halves; lanes 1, 2, 5, and 6 were incubated at 34°C, and lanes 3, 4, 7, and 8 were incubated at 39.6°C in 1% serum. The levels of luciferase RNA (48 h after transfection) are shown for each assay after 20 and 30 cycles of amplification. (A) Cells transfected with the IGF luc plasmid (*Sau*3A promoter [Fig. 1] driving the luciferase gene); (B) cells transfected with the IGF1 luc2 plasmid (*Sma*I promoter [Fig. 1] driving the luciferase gene). Lanes: 1 to 4, BALB/c3T3 cells; 5 to 8: BALB58 cells (stably transfected with the *ts* SV40 T antigen). Amplimers and probes for luciferase are given in Methods and Materials.

of neighboring regulatory elements. We therefore used, to confirm these results, a transient expression assay in which clone L BALB 58 cells were transfected with the IGF luc plasmids (Fig. 1). We used the IGF luc plasmid and another plasmid, with a longer rat IGF-1 promoter (~1.2 kb) still driving the luciferase cDNA (IGF luc 2 plasmid; see Fig. 1 and Methods and Materials). 3T3 cells were used as controls. Single pools (for each plasmid and cell line) of transfected cells were then divided into two halves, each half incubated at either 34 or 39.6°C. Luciferase RNA levels, 48 h after transfection, were determined by RT-PCR (Fig. 9). The temperature had no effect on the transient expression of IGF luc (Fig. 9A) and IGF luc2 (Fig. 9B) in 3T3 cells (lanes 1 to 4); however, in BALB 58 cells, luciferase expression was sharply increased at 34 versus 39.6°C (lanes 5 to 8). The difference between the two temperatures in BALB 58 cells was even more striking when the longer IGF-1 promoter (Fig. 9B) was used.

DISCUSSION

The *ts*A58 mutant of SV40 has a point mutation at position 3505 resulting in a Ala to Val change at position 438 of the large T antigen (6). The protein is normal at 34°C, displaying all the functions of the wild-type SV40 T antigen. At 39 to 39.6°C, the protein is incapable of transforming cells (49), of stimulating growth in low serum (see, for instance, this paper), of binding to DNA or the p53 and retinoblastoma gene products (41), and is abnormally phosphorylated (13 [which also gives appropriate references]). The mutation in *ts*A58 can be complemented by other mutations even in distant amino acids of the T protein, indicating that the mutation results in a drastic conformational change at the restrictive temperature (6), which may explain the broad functional failure of *ts*A58 at 39 to 39.6°C. We have used this *ts*A mutant in an expression plasmid, carrying only the T antigen coding gene, to study the effect of T antigen on growth factor requirements of 3T3 cells. The gene also codes for small t, but Ozer and coworkers have shown that small t is dispensable, at least for the functions described in this paper (37). In most experiments, we have used stably transfected cell lines. The *ts* property of T antigen allows us to carry out all experiments in the same cell line at two temperatures, without interference from clonal variability or position effect. Indeed, although we have concentrated on two clones, the validity of our conclusions is confirmed by several findings: (i) several clones of *ts*A58-transformed 3T3 cells displayed the same growth characteristics of clones A and L; (ii) pA6 cells are a different clone, generated by transfection of *ts*A58 into p6 (not 3T3) cells; (iii) similarly, luc A58 cells were also generated from a different clone of 3T3 cells, luc A cells; and (iv) the effect of SV40 T antigen could also be detected in transient expression assays, from single pools of transfected cells. Finally, similar results have been obtained in young WI-38 human fibroblasts (44a).

In all the experiments reported in the literature, including those of Jat and Sharp (22), who used rat cells, the restrictive temperature (39 to 39.6°C) effectively eliminates the function of SV40 large T antigen. For the specific purpose of this paper (growth factor requirements in cells expressing large T), our experiments confirm that the ability to lower such requirements is abrogated in cells incubated at 39.6°C. On the basis of these requisites, our findings conclusively demonstrate that (i) transformation by SV40 T antigen results in a marked increase in IGF-1 mRNA levels and IGF-1 secretion; (ii) this increase abrogates the requirement for IGF-1 in the medium, as in the case of c-*myb* (52); (iii) an antisense oligodeoxynucleotide to the IGF-1 receptor RNA inhibits the growth stimulatory capacity of T antigen; (iv) in cells expressing a functional T antigen, the IGF-1 R is constitutively phosphorylated in SFM; and (v) the increase in the expression of IGF-1 mRNA is, at least in part, due to a transcriptional mechanism.

SV40-transformed 3T3 cells still require PDGF (or 1% serum). Perhaps, PDGF is needed to increase the number of

IGF-1 receptors. Indeed, it has been known for several years that PDGF (and also EGF) increases the number of IGF-1-binding sites by a factor of 2.0- to 2.5-fold (10–12). In turn, an increased number of IGF-1 receptors is necessary for IGF-1 to stimulate cell growth (35, 36). As long as the number of IGF-1 binding sites remains low, as in quiescent 3T3 cells, IGF-1 alone fails to elicit a growth response (17, 35). This hypothesis is supported (i) by our finding that p6A cells overexpressing the human IGF-1 receptor and the SV40 T antigen no longer need PDGF at 34°C but grow in SFM and (ii) by the report of Zilberstein et al. (56), who, although using a different *ts*A mutant of SV40 T antigen, did not find any difference at either permissive or restrictive temperature in the number of IGF-1 binding sites. p6 cells grow in SFM plus IGF-1 and, under these conditions, neither the PDGF nor the EGF receptor is activated (35). There is, however, an alternative explanation; it is possible that, when the IGF-1 receptor is markedly overexpressed, the IGF-1 signal may lose some of its specificity and trigger PDGF-related events. Indeed, in p6 cells, IGF-1 induces c-*myc* mRNA (48), an event that is usually attributed to PDGF.

There is no doubt that SV40 T antigen causes a marked increase in the amount of IGF-1 mRNA; we repeated the experiment several times with BALB 58 cells in two different clones and also in p6A and luc A58 cells. Every time, IGF-1 RNA was not detectable or markedly decreased by RT-PCR at 39.6°C (under the conditions used) but was abundant in cells incubated at 34°C (Fig. 4 and 8). While RT-PCR is only semiquantitative, the differences in IGF-1 mRNA levels at the two temperatures were invariably dramatic. A more modest increase had been reported by Zilberstein et al. (56), but they used a different mutant of SV40 large T, *ts*A209. The increased expression of IGF-1 mRNA is reflected in the secretion of abundant IGF-1 in the medium: when one considers that 10 to 20 ng of IGF-1 per ml in a single dose is sufficient to stimulate growth of cells expressing the proper number of IGF-1 binding sites, a steady concentration of 6 to 10 ng/ml seems to be optimal. The presence of sufficient IGF-1 in the medium of BALB 58 cells at 34°C is also supported by the finding that in p6A cells growing in SFM, at 34°C, the IGF-1 receptor is constitutively autophosphorylated, while it is not in p6 cells in SFM. It would have been desirable to determine the autophosphorylation status of the endogenous IGF-1 receptor in clones A and L of BALB A58 cells: unfortunately, a combination of low IGF-1 receptor expression and the fact that the antibody is anti-human IGF-1 receptor resulted in too weak a signal for a reliable interpretation.

It is also significant that SV40 T antigen-transformed 3T3 cells require the IGF-1 receptor for growth in 1% serum; an antisense oligodeoxynucleotide to the IGF-1 receptor RNA inhibits their growth just as it inhibits IGF-1-mediated growth of p6 cells (35) or the proliferation of T lymphocytes stimulated with phytohemagglutinin and interleukin-2 (40). The SV40 T antigen, therefore, joins a list of agents whose ability to stimulate growth requires the IGF-1/IGF-1 receptor pathway and which includes growth factors like PDGF and EGF (10, 11, 36) and interleukin-2 (40), proto-oncogenes like c-*myb* and c-*myc* (39, 52), and hormones like growth hormone (54) and estrogens (32, 34, 46). Some hemopoietic cell lines grow in and require IGF-1 for growth (15, 20, 31, 33). Other findings suggesting an important role of IGF-1 and its receptor in growth regulation are the following: meiosis in *Xenopus* oocytes is regulated by IGF-1 receptors (21, 43), and the levels of cdc2 mRNA in fibroblasts are also regulated by IGF-1 (48); overexpression of IGF-1 Rs allows NIH 3T3 cells to grow in soft agar (24), and the transforming gene of the avian sarcoma virus UR-2 (v-*ros*) is equally homologous to IGF-1 and insulin receptors, which are in fact more similar to each other. Goldring and Goldring (18), in a review, list the various cell types whose growth is stimulated by IGF-1: keratinocytes, smooth muscle cells, chondrocytes, osteoblasts, and others.

As to the mechanism by which SV40 T antigen increases the expression of IGF-1 mRNA, the data of our Fig. 8 and 9 indicate a transcriptional component. However, the effect of SV40 T antigen on IGF-1 mRNA levels is more striking with the endogenous IGF-1 gene than with the IGF-1 promoter/luciferase constructs. One possibility is that the difference may be due to the fact that the rat IGF-1 promoters we used are short ones, from only about 200 bases to ~1.2 kb; in other words, they could be incomplete promoters. Alternatively, introns and 5′ and 3′ untranslated regions may play a role in determining IGF-1 RNA levels. These possibilities are presently being examined; for the moment, we can say that a transcriptional component is present in the process. This by no means implies that T antigen binds directly to the IGF-1 promoter; the mechanism is probably indirect, although this is another question that has to be resolved. In this respect, Beard et al. (4), in reporting that SV40 activates transcription from the transferrin receptor promoter, present some evidence that similar proteins bind to the SV40 late promoter and to the transferrin promoter.

We are also not implying that the activation of the IGF-1/IGF-1 receptor pathway is the only function of SV40 T antigen. Indeed, we have limited our studies to the effect that a functional T antigen has on growth factor requirements, and certainly other functions of T are needed for transformation. But our findings do explain the lower growth factor requirements of SV40-transformed cells and are also compatible with the ability of SV40 (and other DNA oncogenic viruses) to induce preferentially late growth-regulated genes (25, 30). Our new data are also compatible with our recent report that SV40 T antigen may (indirectly) affect RNA processing (25), since IGF-1 acts largely (but not exclusively) at a posttranscriptional level (8, 57). For the moment, together with the data from the literature, we can conclude that (i) the IGF-1 receptor plays a central role in cell proliferation and its activation is a required step in growth stimulation of 3T3 cells by T antigen and (ii) SV40 T antigen can regulate the expression of IGF-1 and IGF-1 mRNA, a regulation at least partially dependent on transcriptional mechanisms.

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