Inhibition of Simian Virus 40 T-Antigen Expression by Cellular Differentiation

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Murine 3T3T stem cells transfected with pSV3neo DNA were employed to study the effects of somatic cell differentiation on simian virus 40 (SV40) T-antigen expression. This experimental approach was used because the 3T3T cell line is a well-characterized *in vitro* adipocyte differentiation system and the pSV3neo plasmid contains the early region of the SV40 genome and a selective marker, G418 resistance. Cell clones containing stably integrated pSV3neo which expressed T antigen were isolated in G418-containing medium. Most of these cell clones differentiated poorly. However, several clones retained the ability to efficiently differentiate into adipocytes, and with these cell clones, it was established that adipocyte differentiation markedly repressed T-antigen expression. The differentiation-specific repression of T-antigen expression did not result from a loss of proliferative potential associated with terminal differentiation, because it was observed in adipocytes that could be restimulated to proliferate. In such cells, restimulation of cell growth induced reactivation of T-antigen expression. Repression of T-antigen expression was also demonstrated during differentiation of SV40 T-antigen-immortalized human keratinocytes. These results establish that the process of cellular differentiation can repress T-antigen expression in at least two distinct biological systems.

During the process of differentiation, numerous changes in cellular gene expression occur. Since viral genes are dependent on certain host factors for their expression, differentiation can also affect the expression of viral genes. For example, in undifferentiated embryonal carcinoma cells (14) and pluripotent mouse embryonic cells (44), the genes of several oncogenic viruses are expressed poorly or not at all. In contrast, proficient transcription occurs if the viral DNA is introduced into these cells after differentiation. This change in the ability of viral genes to be expressed is thought to involve the loss of diffusible repressor molecules during differentiation which were present previously in the undifferentiated cells. These factors do not appear to be specific because a wide variety of tumor viruses fail to replicate in undifferentiated embryonal carcinoma cells (26, 35, 37), and promoter or enhancer regions of simian virus 40 (SV40) (30), polyomavirus (13, 31), murine leukemia virus (10), and adenovirus type 5 E1A (15) can each compete for these putative negative regulatory factor(s). However, the biological events that occur when pluripotent embryonal cells differentiate are thought not to be representative of regulatory processes involved in control of somatic cell differentiation. This conclusion is supported by observations that there is active transcription of the SV40 early region and other viral genes in undifferentiated somatic cells in culture (19, 24). In fact, the expression of some viral genes actually inhibits somatic differentiation in certain cells (4, 38).

To determine if somatic cell differentiation can modulate T-antigen expression, 3T3T cells were transfected with pSV3neo DNA and selected for resistance to the antibiotic G418. Although 3T3T cells normally differentiate very efficiently into adipocytes, most pSV3neo-transfected cells differentiated very poorly. Nevertheless, some cell clones were isolated which differentiated well. One clone, CSV3-1, could in fact differentiate into adipocytes at a frequency equal to that of the parental 3T3T cell line. In CSV3-1 and 12 other

pSV3neo-transfected clonal cell lines, T-antigen expression was found to be markedly repressed during adipocyte differentiation. The repression of T antigen was reversible when CSV3-1 adipocytes were stimulated to grow and was therefore not a result of terminal differentiation. In addition, the repression of T antigen was evident during the differentiation of SV40 T-antigen-immortalized human keratinocytes.

MATERIALS AND METHODS

Cell culture. 3T3T murine mesenchymal stem cells were derived from BALB/c 3T3 clone A31 cells (7) and were cultured as previously described (16, 25, 42). Briefly, 3T3T cells and pSV3neo-transfected derivative cell clones (see below) were grown in Dulbecco modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS). Cultures were maintained at subconfluent cell densities and were mycoplasma-free. The HE-SV cell line, an SV40 T-antigen-immortalized human keratinocyte cell line, was kindly provided by P. M. Howley (2). HE-SV cells were cultured in serum-free MCDB 153 medium containing various cofactors and growth factors (41).

DNA transfection and isolation of cell clones expressing T antigen. Exponentially growing 3T3T cell cultures were transfected with pSV3neo (33) at 5 µg of DNA per 2.5×10^5 cells, using a standard calcium phosphate technique (9). Cells with stable chromosomal integration of this plasmid were selected by culturing in medium containing 500 µg of G418 per ml for ≥ 4 weeks (5, 6). G418-resistant cells were placed in differentiation-inducing conditions described below, and after 7 days, single adipocytes and single undifferentiated cells were isolated from the same dishes and cloned by using the microchip method described previously (34). Clonal lines were developed from these individual cells by inducing cell proliferation with DMEM containing 30% FBS and 50 µg of insulin per ml.

The HE-SV cell line was derived previously from human keratinocytes after transfection with plasmid DNA containing the SV40 early region by Banks-Schlegel and Howley (2).

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This cell line is immortal, expresses SV40 T-antigen, and retains the potential to differentiate.

Procedures for differentiation induction. To induce adipocyte differentiation in 3T3T and pSV3neo-transfected 3T3T cells, exponentially growing monolayer cultures were dissociated into single cells with 0.1% EDTA in phosphate-buffered saline (PBS). These cells were plated at a density of 5×10^3 cells per cm^2 on bacteriological plastic petri plates which had not been tissue culture treated or gamma irradiated. Such cells were cultured in heparinized DMEM containing 25% human plasma or 25% CEPH, which is a plasma fraction that induces high-frequency adipocyte differentiation (42, 45). The degree of adipocyte differentiation was monitored over a 5- to 14-day interval by microscopic visualization of lipid accumulation (16).

To induce differentiation of SV40 T-antigen-immortalized human keratinocytes, HE-SV cells were dissociated from monolayer cultures with 0.25% trypsin and placed in suspension over an agarose layer in standard culture medium for 4 days. Differentiation was monitored by immunofluorescence analysis with an antibody directed against a differentiation-specific protein, involucrin (1, 21).

Transformation assays. Two in vitro assays which are markers for tumorigenicity in murine systems (28) were used to establish the transformed phenotype of pSV3neo-transfected 3T3T cells. One assay measures the potential for cells to grow in multilayers, which correlates with the loss of contact inhibition, and the other measures colony formation in soft agarose, which correlates with anchorage-independent growth. To determine multilayer growth potential, cells were cultured as monolayers for 4 weeks postconfluence with repeated refeeding of DMEM plus 10% FBS. These cultures were then fixed and stained to visualize type III foci (22), which consist of multilayer cell colonies. Growth in soft agarose was determined by suspending cells in 0.4% agarose and placing the suspension over 0.8% agarose in 6-well plates at 10^4 cells per well. The agarose solutions were made in DMEM plus 10% FBS, and the wells were repeatedly refed DMEM plus 10% FBS every 2 to 3 days. Transformed colonies were assayed within 10 to 14 days (18, 20). Control 3T3T cells did not form colonies of >8 cells in soft agarose, while T-antigen-transformed 3T3T cells formed large colonies of >200 cells.

T-antigen expression assays. T-antigen expression was monitored by indirect immunofluorescence, immunoprecipitation, and Western blot (immunoblot) analyses using a monoclonal antibody, Ab-1 (Oncogene Science), which recognizes an epitope shared by SV40 large T and small t antigens (11). Standard techniques were used (36, 40) and the instructions of the supplier were followed. Identical results were obtained with another monoclonal antibody, Ab-2 (Oncogene Science), which recognizes an antigenic region of the large T antigen which is not shared by small t antigen (11). A specific fixation procedure for immunofluorescence was utilized to optimize T-antigen staining and to preserve the adipocyte morphology. Cells were fixed for 1 min in 3.7% formaldehyde in PBS, permeabilized 60 to 90 s in 0.5% Triton X-100 in PBS, fixed again for 10 to 15 min in 3.7% formaldehyde in PBS, treated 2 min with -20°C methanol, and then washed three times with PBS. These fixed cells were incubated with the Ab-1 or Ab-2 monoclonal antibody. After rinsing, cells were incubated with a fluorescein-conjugated anti-mouse immunoglobulin G (IgG) antibody (Organon Teknica) and then stained with Hoechst 33258 to visualize the nuclei. In this study, all cells we refer to as T antigen positive displayed typical nuclear staining. The

HE-SV cells were fixed by using the same fixation method; however, during the incubation with the T-antigen antibody, a rabbit anti-human involucrin antibody (Biomedical Technologies, Inc.) was also added. Consequently, a rhodamine-conjugated anti-rabbit IgG antibody (Organon) was added with the fluorescein-conjugated anti-mouse IgG second antibody incubation; therefore involucrin was visualized by using the rhodamine filter set, and T antigen was visualized by using the fluorescein filter set.

As stated above, the Ab-1 and Ab-2 monoclonal antibodies were also used for immunoprecipitation (36) and Western blot analysis (40) by the standard techniques described by the suppliers. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed using 10% polyacrylamide gels (17) for both techniques.

Southern blot analysis. Southern blot analysis was performed by standard techniques (32). Genomic DNA was isolated as described by Blin and Stafford (3) and was digested with restriction enzymes (Boehringer Mannheim) according to the supplier's instructions. The DNA probes, pSV3neo or the 3.5-kilobase (kb) *Bam*HI fragment containing the SV40 early region, were uniformly labeled with [^{32}P]dCTP (Dupont, NEN Research Products) by using a standard nick-translation procedure according to the supplier's instructions.

RESULTS

The 3T3T adipocyte differentiation model system (7) was chosen for the majority of the work in this report, because the biological parameters that control their proliferation and differentiation have been well characterized (16, 25, 42). Briefly, three steps are involved in the process of adipocyte differentiation under the conditions used here. First, cells enter a distinct predifferentiation state in which they are reversibly growth arrested and do not exhibit an adipocyte phenotype. Thereafter, these growth-arrested cells differentiate into adipocytes that retain the ability to be restimulated to proliferate. Adipocytes at this nonterminal state of differentiation can finally advance to a state of terminal differentiation.

Isolation of SV40 T-antigen-transformed cell clones. To isolate T-antigen-transformed cells, 3T3T cells were transfected with pSV3neo DNA, a plasmid containing the SV40 early region sequences which encodes large T and small t antigens, along with a gene conferring resistance to the neomycin analog G418 (33). After being cultured with G418, resistant cell populations that had stably integrated pSV3neo were isolated and found to be T antigen positive by immunofluorescence. These cells were transferred to the most stringent differentiation-promoting conditions we have developed which minimize growth and enhance adipocyte differentiation (45; see Materials and Methods). By using these stringent culture conditions, $\geq 95\%$ of nontransfected 3T3T cells undergo adipocyte differentiation within 5 to 7 days. In contrast, populations of pSV3neo-transfected cells that were G418 resistant differentiated to only 1 to 3% of control 3T3T levels, similar to previous reports using different conditions (4, 38).

In an attempt to isolate cell clones that expressed T antigen and retained the ability to differentiate at high frequency, single adipocytes were isolated from the pSV3neo-transfected cell populations that were cultured in a differentiation-inducing environment. Cell clones were generated by restimulating single adipocytes to reenter the cell cycle, divide, and dedifferentiate by treatment with DMEM

TABLE 1. Biological characteristics of pSV3neo-transfected 3T3T cells

Cell	No. of clones	% Differentiation ^a	G418 resistance ^b	T-antigen expression ^c	Transformation characteristic ^d shown by growth in:		
					Soft agarose	Multilayer	Low serum
3T3T (control)		>95	—	—	—	—	—
pSV3neo-transfected cells							
G418-resistant population		1–3	+	+	+	+	+
G418-resistant cell clones derived from:							
Undifferentiated cells	5	1–4	+	+	+	+	+
Differentiated cells	7 ^e	1–5	+	+	+	+	+
	4	10–15	+	+	+	+	+
	1	30–40	+	+	+	+	+
	1 ^f	>95	+	+	+	+	+

^a The percent differentiation was determined microscopically as the percentage of adipocytes after culturing the cells under differentiation-inducing conditions for 7 days.

^b Resistance to G418 was defined as growth in medium containing 500 µg of geneticin per ml.

^c Determined by immunofluorescence as described in Materials and Methods.

^d See Materials and Methods for details.

^e CSV3-14 cell clone is from this group.

^f CSV3-1 cell clone.

containing 30% FBS and 50 µg of insulin per ml (43). After a few population doublings, all such cell clones returned to an undifferentiated state and thereafter were maintained as exponentially growing cultures. As controls, undifferentiated transfected cells from the same cultures were cloned under identical conditions.

Table 1 summarizes some biological characteristics of the pSV3neo-transfected 3T3T cell clones. All cell clones were resistant to G418, expressed T antigen, and therefore presumably had integrated at least one functional copy of pSV3neo plasmid. All clones also expressed a transformed phenotype as characterized by their growth in soft agarose, multilayers, and medium containing 0.5% serum; these three transformation assays measure anchorage-independent growth, loss of contact inhibition, and low growth factor requirements, respectively. These transformed clones were further analyzed to determine if they could redifferentiate. While only minimal differentiation could be induced in 5 clones derived from adipocytes, ≥10% of the cells could be induced to differentiate (Table 1). One of these cell clones, CSV3-1, retained the ability to differentiate at a frequency equal to that of the parental 3T3T cells. This clone will be further characterized below and was the main cell line used to study the effects of adipocyte differentiation on T-antigen expression. Another cell clone, CSV3-14, differentiated poorly and was used as a control.

Characterization of the CSV3-1 and CSV3-14 cell clones.

The CSV3-1 and CSV3-14 clones were examined first to determine if any correlations existed between pSV3neo gene copy number, T-antigen expression, and differentiation potential. These clones were chosen because preliminary data indicated that CSV3-1 demonstrated low T-antigen expression and high differentiation potential, whereas CSV3-14 demonstrated high T-antigen expression and low differentiation potential. High-molecular-weight genomic DNA was isolated from CSV3-1 and CSV3-14 cells and examined by Southern blot analysis. After digestion with either *Eco*RI or *Bam*HI, equal amounts of genomic DNA from each clone were analyzed by agarose gel electrophoresis, Southern blotting, and probing with ³²P-labeled pSV3neo DNA. *Bam*HI cleaves twice and *Eco*RI cleaves once within the pSV3neo plasmid. Multiple bands were evident for both cell clones when using either *Eco*RI (Fig. 1, lanes a and b) or

*Bam*HI (lanes c and d). Therefore, it is probable that pSV3neo integrated into more than one site within the genome in both clones. When comparing the signal intensities for both the *Eco*RI and *Bam*HI digests, it is apparent that CSV3-1 cells have more copies of integrated pSV3neo than does CSV3-14. It is likely that the large amounts of pSV3neo in CSV3-1 cells is due to concatemer formation during transfection (note the strong band corresponding to a 9.1-kb *Eco*RI fragment in lane a). Although CSV3-1 apparently integrated many more copies of pSV3neo DNA, the amount of intact SV40 early region insert appears to be comparable with that found in CSV3-14. This is illustrated by the approximately equivalent intensities of a 3.5-kb fragment in the *Bam*HI DNA digests of both cell clones

FIG. 1. Southern blots of DNA from CSV3-1 and CSV3-14 cells. DNA (10 µg) from cell clones CSV3-1 (lanes a, c, and e) and CSV3-14 (lanes b, d, and f) were digested with *Eco*RI (lanes a and b), *Bam*HI (lanes c and d), or *Eco*RI and *Bam*HI (lanes e and f) and loaded into individual lanes of a 1% agarose gel. A Southern blot was made from this gel, and the nitrocellulose was probed with ³²P-labeled DNA from pSV3neo (lanes a through d) or 3.5-kb SV40 early-region *Bam*HI fragment (lanes e and f). *Bam*HI cleaves intact pSV3neo plasmid twice, generating a 5.6-kb fragment of the original pSV2neo plasmid and a 3.5-kb fragment representing the SV40 early-gene insert. *Eco*RI cleaves only once within the pSV3neo plasmid. The arrowhead indicates the migrational distance of a 3.5-kb DNA fragment. Positions (in kilobases) of molecular size markers are shown to the left of the gel.

FIG. 2. (A) Immunoprecipitation studies to determine T-antigen synthesis. After 1 h of incubation in [35 S]methionine, extracts from exponentially growing control 3T3T, CSV3-1, or CSV3-14 cells were immunoprecipitated by using T-antigen-specific monoclonal antibody Ab-1 and analyzed by SDS-polyacrylamide gel electrophoresis. (B) Western blot analysis for T-antigen abundance. Cell extracts from 5×10^5 cells from control 3T3T, CSV3-1, and CSV3-14 exponentially growing cultures were separated by SDS-polyacrylamide gel electrophoresis and Western blotted. The blot was probed with Ab-1. T, Position to which control T antigen migrated.

(*Bam*HI cleaves a 3.5-kb fragment from pSV3neo which contains the entire SV40 early-gene insert). The relative number of intact SV40 early-region copies was further analyzed for CSV3-1 and CSV3-14 by probing *Eco*RI-*Bam*HI genomic DNA digests with labeled DNA from only the SV40 3.5-kb insert (lanes e and f). When probing specifically for SV40 sequences, the intensity of intact 3.5-kb SV40 early-gene sequences was again found to be equivalent in CSV3-1 and CSV3-14 genomic DNA.

As stated above, preliminary data suggested that during exponential growth CSV3-14 cells display a more intense T-antigen immunofluorescent image than CSV3-1 cells. This would indicate that T antigen was more abundant in CSV3-14, and yet the gene copy number for the SV40 early region in CSV3-14 appears to be comparable with that found in CSV3-1. Therefore, the synthesis and abundance of T antigen was assayed for these two cell clones. When T antigen was immunoprecipitated after a 1-h pulse of [35 S]methionine, synthesis of T antigen was found to be considerably higher in CSV3-14 cells than in CSV3-1 cells (Fig. 2A), consistent with the preliminary immunofluorescence data. Both cell clones clearly synthesized a protein, recognized by a T-antigen specific monoclonal antibody, which could not be detected in control nontransfected 3T3T cells. Western blot analysis (Fig. 2B) confirmed that T-antigen steady-state levels were also higher in CSV3-14 cells when compared with those of CSV3-1 cells. Once again the antigenic proteins gave the correct size and band patterns on SDS-polyacrylamide gels representative of intact T antigen.

Therefore, although CSV3-1 and CSV3-14 appeared to have similar intact-T-antigen-gene copy numbers, the expression of T antigen was markedly higher in the CSV3-14 cell clone. Additional Southern analyses with several other restriction enzymes detected no genetic rearrangements in the 3.5-kb SV40 early regions of CSV3-1 or CSV3-14 (data not shown). There could be many explanations for the lower T-antigen expression in CSV3-1 cells, such as chromosomal

FIG. 3. T-antigen synthesis is repressed by adipocyte differentiation. CSV3-1 and CSV3-14 cells were cultured in differentiation-inducing conditions, and cell extracts were immunoprecipitated after 1 h of incubation in [35 S]methionine. By day 7, CSV3-1 cultures were 95% adipocytes and remained adipocytes through day 14. CSV3-14 cultures differentiated poorly ($\leq 5\%$) even by day 14 and were proliferating at a slow rate.

position effects on the inserted plasmid DNA. It is also plausible that the large amount of partial SV40 DNA sequences integrated within the CSV3-1 genome can compete for and titrate positive transcription factors necessary for T-antigen expression of the intact SV40 genes.

Inhibition of T-antigen expression by murine adipocyte differentiation. To investigate the effect of differentiation on T-antigen expression, CSV3-1 cells were cultured in differentiation-inducing conditions for 7 days. During this interval, $\geq 95\%$ of CSV3-1 cells expressed the adipocyte phenotype. The rate of T-antigen synthesis, assayed by immunoprecipitation, was markedly repressed in the CSV3-1 adipocyte at day 7 and the rate of T-antigen synthesis at day 14 was almost undetectable (Fig. 3). In contrast, CSV3-14 cells which differentiated poorly when cultured under identical differentiation-inducing conditions expressed high levels of T antigen (Fig. 3). Therefore, in the absence of differentiation, repression of T-antigen expression does not occur.

The abundance of T antigen was also measured during differentiation of CSV3-1 cells by Western blot analysis. The intensity of T-antigen staining decreased progressively, coincidental with adipocyte differentiation (Fig. 4). On days 1 and 2 (Fig. 4, lanes b and c), when no differentiation was evident, T-antigen abundance was equal to that found in exponentially growing CSV3-1 cells (Fig. 4, lane a). The levels of T antigen detected by Western blot decreased between days 3 and 9 as the percentage of cells expressing the adipocyte morphology increased (Fig. 4, lanes d through h). By day 12 (Fig. 4, lane h) when differentiation was

FIG. 4. T-antigen abundance is repressed during adipocyte differentiation. Western blot analysis was performed on cell extracts from 4×10^5 CSV3-1 cells on days 0, 1, 2, 5, 6, 7, 11, and 12 (lanes a through h, respectively) in differentiation-including conditions. Blots were probed with T-antigen specific monoclonal antibody Ab-1.

maximum, the T-antigen levels were only marginally detectable.

The data from immunoprecipitation and Western blot analyses indicated that the rate of synthesis and abundance of T antigen are markedly repressed during adipocyte differentiation. However, these biochemical studies could not establish whether the rare undifferentiated cells in the CSV3-1 adipocyte cultures continued to express T antigen as predicted if the repression of T antigen was differentiation specific. These studies also could not determine if T-antigen expression was repressed in adipocytes of other pSV3neo-transfected 3T3T cell clones which differentiated poorly. To overcome this limitation, immunofluorescence analysis was performed. There was some cell-to-cell variability in fluorescence intensity. However, essentially all exponentially growing cells exhibited typical T-antigen immunofluorescence (Fig. 5C). The results showed that during CSV3-1 differentiation, nuclear T-antigen staining was reduced to undetectable levels when cells visibly displayed an adipocyte phenotype (Fig. 5f). In contrast, undifferentiated cells in the same environment continued to express T antigen (Fig. 5f). In addition, immunofluorescence analysis of T-antigen expression was performed on the other 12 clones shown in Table 1. Although none of the other clones differentiated as well as CSV3-1, repression of T-antigen expression was observed in all these cell clones as they underwent adipocyte differentiation, while undifferentiated cells in the same cultures continued to show T-antigen immunofluorescence. Immunofluorescence analysis also demonstrated that repression of T-antigen expression was not dependent on the human plasma factors normally used to induce differentiation. This was demonstrated by culturing CSV3-1 cells at a high cell density in DMEM plus 10% FBS, in which some cells were able to differentiate spontaneously into adipocytes at a low frequency (<1%). T-antigen immunofluorescence was repressed in these adipocytes even though these cells had not been exposed to human plasma.

It was also found that repression of T-antigen expression is not a consequence of terminal differentiation. This was established by showing that after 14 days of culture in differentiation-promoting conditions, when cell division had stopped and T antigen had been repressed for 8 to 10 days, >90% of the CSV3-1 adipocytes could be induced to reinitiate proliferation. Soon after restimulation of cell proliferation, T antigen was reexpressed and could be detected by Western blot analysis (Fig. 6). Figure 6 also illustrates the reappearance of high steady-state levels of cellular p53, which is stabilized by functional T antigen (12).

Inhibition of T-antigen expression by human keratinocyte differentiation. The observations reported above demonstrate that nonterminal adipocyte differentiation induces an activity which represses SV40 T-antigen expression. To determine whether this phenomenon is unique to murine adipocyte differentiation, similar studies evaluated the expression of SV40 T antigen during differentiation of HE-SV cells. HE-SV is a cell line of SV40 T-antigen-immortalized human keratinocytes, which can be induced to undergo squamous differentiation when cultured in suspension (2). The results of this study showed that HE-SV cells also repressed T-antigen immunofluorescence as the expression of a differentiation-specific marker, involucrin (1, 21), became detectable (results not shown). All undifferentiated HE-SV cells (involucrin negative) showed bright nuclear T-antigen staining. In contrast, immunofluorescence analysis demonstrated that T antigen was undetectable in 60% of the involucrin-positive differentiated HE-SV cells, and was

FIG. 5. Repression of T-antigen expression in adipocytes demonstrated by immunofluorescence. Cells from CSV3-1 cultures in exponential growth (A, B, and C) and CSV3-1 cultures in differentiation-inducing conditions (D, E, and F) show the differentiation specificity of T-antigen repression. Phase-contrast microscopy shows cell morphology (A and D). Nuclei are visualized with Hoechst 33258 (B and E). T-antigen nuclear staining is shown by immunofluorescence by using the Ab-1 antibody (C and F). Note the one rare undifferentiated cell in the adipocyte culture; this cell retains the T-antigen nuclear staining while the adipocytes are all T antigen negative.

markedly decreased in most of the remaining cells that expressed involucrin. That is, T-antigen expression was repressed either partially or totally in 85 to 90% of the differentiated HE-SV cells.

DISCUSSION

In this study, SV40 T-antigen expression was repressed during the differentiation of both murine adipocytes and human keratinocytes in vitro. In murine 3T3T cell clones that retained the ability to differentiate after stable transfection with pSV3neo DNA, differentiation was uniformly accompanied by repression of T-antigen expression when

FIG. 6. T-antigen reactivation when adipocytes are restimulated to proliferate. Extracts of 5×10^5 CSV3-1 cells in exponential growth, of adipocytes after 12 days in differentiation medium, and of adipocytes after 48 h of proliferation restimulation (30% FBS and 50 μ g of insulin per ml) were evaluated by polyacrylamide gel electrophoresis and blotted before an incubation with two monoclonal antibody probes, one specific for T antigen and the other specific for p53. The abundance of both proteins is repressed in adipocytes but returns after growth restimulation.

evaluated by immunofluorescence. In one such clone, CSV3-1, which differentiated as efficiently as 3T3T control cells, the abundance and synthesis of T antigen were also shown to be repressed during CSV3-1 adipocyte differentiation. This differentiation-specific repression of T-antigen expression was not restricted to murine adipocytes, because the process of differentiation also repressed T-antigen expression in a SV40 T-antigen-immortalized human keratinocyte cell line.

Since repression of T-antigen expression was evident in both nonterminally and terminally differentiated adipocytes, it is evident that the repression does not result from a loss of proliferative potential. In fact, T antigen was reexpressed after growth restimulation and dedifferentiation of these adipocytes. Repression of T-antigen expression was also independent of human plasma factors used to induce adipocyte differentiation. This was demonstrated by the repression of T-antigen immunofluorescence in CSV3-1 adipocytes which differentiated at a high cell density in medium devoid of human plasma components. In addition, repression of T-antigen expression was not simply a result of a general reduction in cellular metabolism associated with differentiation for three reasons. First, T-antigen expression was not repressed in undifferentiated growth-arrested cells that show similar metabolic characteristics compared with growth-arrested differentiated cells. Second, undifferentiated cells that were present in cultures intermixed with adipocytes expressed T antigen even though they were typically not dividing as determined by a failure to incorporate [3 H]thymidine into DNA. Finally, when CSV3-1 cells were cultured at a low density in growth factor-deficient medium on poorly adherent surfaces, their growth rate could be markedly reduced without inducing differentiation and yet all such cells displayed levels of T-antigen immunofluorescence comparable with those of exponentially growing cultures. Altogether, the results presented above provide strong evidence that the repression of T-antigen expression is differentiation specific in at least two species and two cell types.

The expression of several viral genes, including the SV40 early region, previously was reported to be suppressed in the undifferentiated pluripotent cells of murine preimplantation embryos (44) and cultured embryonal carcinoma cells (14). However, viral genes introduced into these cells can be efficiently transcribed after these embryonic cells are induced to differentiate into cells expressing markers for more-mature lineages. The observations described in this report appear to be in direct contradiction to those studies, since we demonstrate that murine adipocyte and human keratinocyte differentiation represses T-antigen expression. This apparent discrepancy could be explained by a variety of differences between the experimental systems used in the different studies. Most significant in our opinion is the fact that the embryonal carcinoma cell and related differentiation systems are models of very early embryonic development of pluripotent cells (29), while 3T3T and HE-SV cells are models for somatic cell differentiation. From this perspective, undifferentiated 3T3T and HE-SV cell lines actually represent cells derived from the differentiation of pluripotent cells to somatic cells. Together these observations suggest that the SV40 early region genes and other viral genes are repressed both in early embryonic pluripotent cells and after somatic cell differentiation but that they are not repressed in somatic stem cells or progenitor cells.

The exact mechanisms that might regulate the expression of these viral genes during development or differentiation are not known. Studies using early embryonic cells suggest that although DNA methylation may be involved, negatively acting diffusible factors probably suppress transcription of the SV40 early region (30). In our opinion, it would be unlikely that the repression of T-antigen expression in embryonal carcinoma cells and in differentiated CSV3-1 and HE-SV cells would result from the evolution of a cellular mechanism that is specific for SV40 genes or proteins. It is more probable that repression of SV40 T antigen involves normal mechanisms by which cellular genes are inactivated in early development and during differentiation. Therefore, we are presently investigating how somatic differentiation-induced repression of T-antigen expression is regulated. Djian et al. (8) recently demonstrated that differentiation can suppress the expression of a reporter gene, chloramphenicol acetyltransferase (CAT), under the control of the SV40 early gene regulatory sequences and that the mode of regulation was transcriptional. We have also found probable transcriptional regulation of the SV40 regulatory sequences when linked in a plasmid to the chloramphenicol acetyltransferase reporter gene and that this differentiation-induced repression does not repress chloramphenicol acetyltransferase expression when linked to the Rous sarcoma virus or mouse mammary tumor virus regulatory sequences (C.-Y. Tzen et al., submitted).

A most important aspect of these studies concerns the fact that T antigen can affect many cellular processes that mediate the control of cell proliferation and differentiation (19, 23, 27, 39). Therefore, it is important to determine how T-antigen expression is regulated and if these molecular mechanisms are significant in the process of neoplastic transformation.

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