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SV40 Transformation of Swiss 3T3 Cells Can Cause a Stable Reduction in the Calcium Requirement for Growth

NANCY B. NICHOLSON, SUZIE CHEN, GEORGE BLANCK, and ROBERT POLLACK
Department of Biological Sciences, Columbia University, New York 10027. Dr. Blanck's present address is Department of Biochemistry, Harvard University, Cambridge, Massachusetts 02138

ABSTRACT A well-characterized SV40-transformed Swiss 3T3 line, SV101, and its revertants were tested for the ability to grow in reduced Ca^{++} (0.01 mM). Transformants and revertants did not differ from the parent 3T3 line in their Ca^{++} requirements. All three classes of cells grew less well in low Ca^{++} than in regular Ca^{++} (2.0 mM). SV40 transformants were then selected for the ability to grow in reduced Ca^{++}. This new class of transformants was found to grow in 1% serum, grow in soft agarose, have a reorganized actin cytoskeleton, and express viral T antigens, as well as grow well in low Ca^{++}.

One of the selected clones was found to be T antigen-negative, yet was transformed in the serum, anchorage, actin, and Ca^{++} assays. It is possible that this clone was a spontaneous transformant. However, Southern blot analysis revealed the presence of integrated SV40 DNA. In addition, this analysis revealed the absence of an intact early region fragment, which codes for the viral T antigens. One explanation of this result may be that the mechanism of viral transformation for growth in low Ca^{++} involves viral–host DNA interactions that may not require a fully functional T antigen. In this case SV40 integration may be acting as a nonspecific cellular mutagen.

Calcium ion affects a variety of cellular processes, including cell motility in muscle and nonmuscle cells, axonal flow in neurons, cytoplasmic streaming, ciliary movement, membrane permeability to cations and water, and cell–cell communication through gap junctions (for a review, see reference 28). Extracellular Ca^{++} is also required for cell growth (1, 5). Hormones are required as well for cell growth, and hormonal stimulation of cell division may be mediated through the binding of Ca^{++} and calmodulin (12). The Ca–calmodulin complex regulates a variety of proteins and enzymes (12) in a low background level of intracellular free Ca^{++}, which is maintained by sequestering of Ca^{++} in the mitochondria and other organelles, by pumping through the cell membrane, and by intracellular binding to Ca^{++} buffers such as calmodulin. Cellular Ca^{++} is maintained in three pools: a very fast turnover pool of extracellular membrane-bound Ca^{++}, a fast turnover pool of cytosolic Ca^{++}, and a slow turnover pool of sequestered Ca^{++} (14, 17).

Neoplastic transformation changes many growth properties of cultured cells. For example, the small DNA virus, SV40, causes cultured fibroblasts from mouse, rat, and hamster to partially or entirely lose their growth requirements for serum, anchorage (25), and insulinlike growth factors (Powers, R. S., S. Chen, and R. Pollack, submitted for publication), and alters the organization of the actin cytoskeleton of the cell (24). The transformed phenotypes generated by SV40 can vary considerably. Risser and Pollack (25) have shown that, after SV40 infection of Swiss 3T3 mouse cells, three classes of transformants arose from clones picked without regard to their morphology. Some of these clones were T-negative and serum transformed. Others demonstrated variegated T-antigen expression, but were able to grow in low serum, and to intermediate densities on plastic, but grew poorly without anchorage. The third class of transformants was "fully transformed" in all assays tested.

Such variability in the transformed phenotype is striking considering the simplicity of the SV40 genome. It could arise because of a pleiotropic effect of the viral gene products on the host cell and variation in the host cell population. Omar and Lanks (22) have shown that SV40 transformants derived from mixed populations varied from clone to clone in their ability to grow in low serum, in their saturation density, in cloning efficiency on plastic and in agar, and in the amount of nuclear T antigen, as detected by immunofluorescence, depending on the phenotype of the parent population.

To determine the full extent of the transformed phenotype it is necessary to construct different selective systems. If a transformed phenotype is generated only at a low frequency,

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it will not be observed unless selected. The proper cells in a given selective assay must be untransformed in that assay. For example, if the normal population grows well in 1% serum (such as National Institutes of Health [NIH] 3T3), one cannot select for serum transformation. Thus the full potential of SV40 transformation may not be observed without selection in a suitable normal host cell.

Some transformed phenotypes arise alone, whereas others may be linked to several phenotypes. Risser and Pollack (25) showed that minimally transformed clones were only serum-transformed, whereas full transformants were anchorage-, density-, and serum-transformed. Therefore, transformed clones that have been isolated under selective conditions may carry other transformed phenotypes with them.

The Ca^{++} requirement for growth can be changed by chemical or viral transformation so that the transformants can grow in low Ca^{++} concentration (1, 3, 6, 16). In this paper we examine the Ca^{++} requirement of established 3T3 mouse cells and their transformants and describe the direct selection of calcium-independent transformants after infection by SV40.

MATERIALS AND METHODS

Cell Lines

3T3 R is a subclone of Swiss 3T3 (33). The SV40 transformant SV101 and its revertants, FL2, LS1, LS2, and Aγ4 were previously described (2, 10). NIH 3T3 was kindly provided by M. Wigler (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). SNIH is an SV40 transformant selected in the dense focus assay. NFS and C57L and their SV40 transformants, SN-2 and SC57L have been described (9). All cell lines were free of mycoplasma when tested periodically (11).

Media

All cell lines were maintained in Dulbecco's modified Eagle's medium (Gibco Laboratories, Grand Island, NY), 10% (vol/vol) fetal calf serum (FCS[1]; Gibco Laboratories) (regular growth medium). Defined Ca^{++} medium was prepared by adding $CaCl_2$ to a mixture of Ca^{++}-free Dulbecco's (Gibco Laboratories) and 10% (vol/vol) chelex-treated FCS (7). Chelex-100 was purchased from Bio-Rad Laboratories (Richmond, CA). $CaCl_2$ was added to the defined Ca^{++} media at a concentration of either 0.01 mM or 2.0 mM.

Growth Assay

The relative growth rate of cells in various Ca^{++} concentrations was determined by plating 2×10^4 cells per 35-mm dish in regular growth medium in duplicate dishes. 1 d after plating, the initial cell number was determined using a model Z_b Coulter counter (Coulter Electronics Inc., Hialeah, FL). Sister dishes were rinsed with 0.01 mM Ca^{++} medium and refed with either 0.01 mM or 2.0 mM Ca^{++} medium. 3 and 6 d after plating the cell number was again determined. The relative growth rate (RGR) was calculated as: RGR = doublings in 0.01 mM Ca^{++}/doublings in 2.0 mM Ca^{++}.

Statistical Analysis

Student's *t*-test was used to determine significant differences in the mean RGR between the various cell lines (21). The standard formula for Student's *t*-test was employed, using the following formula for the estimated variance of the mean RGR (15): $s^2_{RGR} = [s^2_{0.01\,mM} + (RGR)^2(s^2_{2\,mM})]/(D_{2\,mM})^2$, where s^2_{RGR} = variance of mean RGR; RGR = mean RGR; $s^2_{0.01\,mM}$ = variance of mean doublings in 0.01 mM Ca^{++}; $s^2_{2\,mM}$ = variance of mean doublings in 2.0 mM Ca^{++}; and $D_{2\,mM}$ = mean doublings in 2.0 mM Ca^{++}. A significance level of $P < 0.05$ was used.

Selection of Low Calcium Transformants

3T3R cells were infected with SV40 at a multiplicity of infection of 100 plaque-forming units/cell for 2 h at 37°C. Mock-infected cells were treated in parallel. The following day the cells were trypsinized and seeded dishes at densities of 10^5 and 10^4 cells/60-mm dish in regular growth medium. 1 d after plating the cells were rinsed once with 0.01 mM Ca^{++} medium and refed with either 0.01 or 2.0 mM medium. The cells were refed two times per week with selective media. Dense foci were isolated 3–4 wk later with cloning cylinders and were expanded and maintained in regular growth medium. Parallel dishes were fixed and stained with 1% crystal violet in ethanol.

Analysis of Transformed Phenotypes

GROWTH IN 1% FCS: The growth rate in 1% FCS relative to 10% FCS was determined in the growth assay as described above.

GROWTH IN AGAROSE: The ability to grow in soft agarose was determined as previously described (20). Cells were plated at densities of 10^3, 10^4, or 10^5 cells/dish in 0.3% agarose over a bed of 0.6% agarose using regular growth medium. After 3 wk, the dishes were scored for colonies >0.2 mm.

ACTIN ORGANIZATION: The actin cable pattern was visualized using rhodamine-phalloidin as previously described (36). The cells were scored in one of four classes of actin organization. These classes range from cells with very large cables throughout the cytoplasm (I) to those with no discernible cables (IV) (36).

IMMUNOPRECIPITATION OF T ANTIGEN: The T proteins were examined as previously described (9).

SOUTHERN BLOT ANALYSIS: Integrated viral DNA was examined by the Southern blot technique as previously described (2, 32, 39).

RESULTS

Ca^{++} Requirement of Normal and SV40-transformed Mouse Cell Lines

We first tested a well-characterized set of mouse cell lines for their Ca^{++} requirements in the RGR assay. The results are shown in Table I. The parental line, 3T3R, had a moderate ability to grow in reduced Ca^{++}; the fully transformed line, SV101, did not have a further reduced Ca^{++} requirement compared with 3T3R. Revertants of SV101 have either regained the serum requirement of 3T3R (Aγ4), or the anchorage requirement (FL2), or both (LS1, LS2) (37, 38). These revertants also showed Ca^{++} requirements similar to those of 3T3R and SV101 (Table I).

Because the fully transformed line, SV101, did not show a reduced Ca^{++} requirement compared with the untransformed parent line, Swiss 3T3R, we tested another mouse line, NIH 3T3 and its SV40 transformant, SNIH. NIH 3T3 was more sensitive to reduced Ca^{++} than was Swiss 3T3R (Table I). SNIH had an RGR in 0.01 mM Ca^{++}. Thus, in this more sensitive cell line, SNIH, calcium transformation can be observed without direct selection.

Two precrisis mouse cell strains and their SV40 transformants were also examined in the growth assay (Table I). The untransformed cell strains, NFS and C57L were both sensitive to reduced Ca^{++}, with RGRs similar to that of NIH 3T3. The transformant of NFS, SN-2, had an RGR that was significantly higher than its parent line, while SC57L had a Ca^{++} requirement that was not significantly different from untransformed C57L (Table I). NFS is an inbred strain of NIH mice (9). It is interesting that both NFS and NIH 3T3 had similar RGRs in low Ca^{++} and exhibited calcium transformation without direct selection.

Direct Selection of Calcium Transformants

Because neither SV101, the "fully transformed" derivative of Swiss 3T3, nor the revertant lines had altered Ca^{++} requirements compared with untransformed 3T3R, we hypothesized that this physiological change characteristic of transformed cells in other systems might not be recovered from Swiss 3T3 after SV40 infection without direct selection. Therefore we

[1] *Abbreviations used in this paper:* FCS, fetal calf serum; RGR, relative growth rate.

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TABLE I

Relative Growth Rate in 0.01 mM Ca^{++}: 3T3R, Mouse Cell Strains, and Their Transformants

Cell line	RGR (SD)*	Transformed phenotype S	Transformed phenotype A‡	*t*-test vs. 3T3R	*t*-test vs. SV101	*t*-test vs. parent line
3T3R	0.39 (0.14)	−	−	—	NS§	NS
SV101	0.42 (0.35)	+	+	NS	—	NS
Aγ4	0.35 (0.45)	−	+	NS	NS	NS
LS1	0.44 (0.089)	−	−	NS	NS	NS
LS2	0.35 (0.32)	−	−	NS	NS	NS
FL2	0.53 (0.29)	+	−	NS	NS	NS
NIH 3T3	0.14 (0.039)	+	−	NS	—	—
SNIH	0.49 (0.11)	+	+	—	—	$0.02 < P < 0.05$
NFS	0.16 (0.067)	−	−	$0.02 < P < 0.05$	NS	—
SN-2	0.55 (0.087)	+	+	NS	NS	$0.02 < P < 0.05$
C57L	0.11 (0.12)	−	−	NS	NS	—
SC57L	0.38 (0.055)	+	+	NS	NS	NS

* *RGR*, average doublings in 0.01 mM Ca^{++}/doublings in 2.0 mM Ca^{++}, measured 6 d after plating; *SD*, standard deviation.
‡ *S*, serum; *A*, anchorage.
§ *NS*, not significant; $P > 0.05$.

TABLE II

Transformation Frequencies

	No. dense foci (SD)* per 10^4 cells	per 10^5 cells	*n*‡
SV40 infected			
Regular medium§	8.3	4.7	1
2.0 mM Ca^{++}	6.4 (5.3)	11.0 (14.1)	3
0.01 mM Ca^{++}	1.8 (1.6)	2.3 (1.6)	3
Mock infected			
Regular medium	0‖	0	1
2.0 mM Ca^{++}	0 (0)	0 (0)	3
0.01 mM Ca^{++}	0 (0)	0 (0)	3

* Dense foci were scored as the number of foci per cells plated; *SD*, standard deviation.
‡ *N*, number of experiments.
§ Medium was regular growth medium: Dulbecco's modified Eagle's medium, 10% FCS; or Ca-free Dulbecco's, 10% chelex-FCS, 2.0 mM $CaCl_2$; or Ca-free DME, 10% chelex-FCS, 0.01 mM $CaCl_2$.
‖ No dense foci were observed in a total of 40 mock-infected plates.

TABLE III

Relative Growth Rate in 0.01 mM Ca^{++}, Calcium Transformants

Cell line	RGR (SD)*	*t*-test vs. 3T3R	*t*-test vs. SV101
3T3R	0.39 (0.14)	—	NS‖
SV101	0.42 (0.35)	NS	—
tx A	0.79 (0.47)	NS	NS
tx A cl 1	0.93 (0.048)	$P < 0.001$	$0.01 < P < 0.02$
tx A cl 2	0.89 (0.11)	$0.001 < P < 0.01$	$0.02 < P < 0.05$
tx B	0.61 (0.33)	NS	NS
tx C	0.80 (0.045)	$0.001 < P < 0.01$	$0.02 < P < 0.05$
tx D	0.66 (0.16)	NS	NS
tx E mixed‡	0.87 (0.31)	$0.02 < P < 0.05$	NS
tx E late§	0.98 (0.056)	$P < 0.001$	$0.001 < P < 0.01$
tx F	0.76 (0.15)	$0.01 < P < 0.02$	NS
tx H	0.70 (0.060)	$0.01 < P < 0.02$	NS
tx I	0.94 (0.16)	$0.01 < P < 0.02$	$0.02 < P < 0.05$
tx J	0.64 (0.051)	$0.02 < P < 0.05$	NS
tx K	0.69 (0.056)	$0.01 < P < 0.02$	NS
tx L	0.64 (0.044)	$0.02 < P < 0.05$	NS
tx M	0.62 (0.079)	NS	NS
tx N	0.62 (0.22)	NS	NS
tx O	0.66 (0.069)	$0.02 < P < 0.05$	NS

* RGR was measured 6 d after plating; *SD*, standard deviation.
‡ Averages of early and late passages.
§ Averages of late passages.
‖ NS = not significant, $P > 0.05$.

next attempted to select SV40 transformants with a reduced Ca^{++} requirement.

3T3R cells were infected with SV40 or mock-infected and then grown in defined Ca^{++} media. The selective media contained 10% chelex-treated FCS and $CaCl_2$ added to a concentration of either 0.01 or 2.0 mM. EGTA was not used to buffer the Ca^{++} in that previous experiments had shown that the relative growth rates were identical when Ca^{++}/EGTA buffers were used or when Ca^{++} was added alone to the media (data not shown). Dense foci of transformed cells growing on a monolayer of untransformed cells appeared in the SV40-infected dishes in ~3–4 wk. The transformation frequency was calculated as the average number of dense foci per number of cells plated. The frequency at which dense foci appeared varied from experiment to experiment, but was generally lower in 0.01 mM Ca^{++} medium than in 2.0 mM Ca^{++} or in regular growth medium (Table II). In the mock-infected plates monolayers of untransformed cells were present, but no dense foci were observed.

The selective media contained FCS, which was treated with chelex-100 to remove the Ca^{++}. This resin also removes Mg^{++} and other cations and this difference in the serum may account for the difference in transformation frequency at 2.0 mM Ca^{++} in the defined Ca^{++} media compared with the regular growth medium. The cells were viable in the chelex-treated FCS, but did not grow as well as in the untreated serum. Therefore the selective media may select for cells with the ability to survive in chelex-treated serum as well as for the

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ability to survive in low Ca^{++}. In most cases the growth rate in regular growth medium was higher than that in 2.0 mM Ca^{++} medium (data not shown).

The inoculation density of 10^4 cells per dish was a more favorable condition than 10^5 per dish because the percentage of transformants was higher in the 10^4 dishes than in the 10^5 dishes. Dense foci were isolated from low Ca^{++} dishes and were tested in the various transformation assays beginning at the third passage.

Reduced Calcium Requirement

Clones from three separate SV40 infections were analyzed for the persistence of a reduced Ca^{++} requirement (Table III). The Ca^{++} requirement was determined by measuring the RGR of the cells in 0.01 mM Ca^{++}. The RGR was calculated 6 d after plating as the number of doublings in 0.01 mM Ca^{++}/number of doublings in 2.0 mM Ca^{++}. Four of the six clones (tx A–tx F) from the first experiment, 1/1 (tx H) from the second experiment, and 5/7 (tx I–tx O) from the third experiment had elevated RGRs in low Ca^{++}. All ten calcium-independent transformants grew better in 0.01 mM Ca^{++} than did the parental line, 3T3R, with RGRs of transformed clones ranging from 0.70 to 0.94. However the calcium-independent transformants with moderate RGRs of 0.64–0.76 were not significantly different from the transformant SV101. When SV40-infected and mock-infected monolayers of 3T3R were tested for growth in low Ca^{++}, 3-wk selection in 0.01 mM Ca^{++} did not cause an increased RGR in low calcium compared with those selected in 2.0 mM Ca^{++} (data not shown).

The phenotype of a reduced Ca^{++} requirement was stable for many passages. This was shown by subcloning and maintaining tx A without selective pressure; the RGRs of subclones A-1 and A-2 increased from 0.79 to 0.93 and 0.89, respectively. Late passage tx E had an RGR of 0.98 compared with an average RGR of 0.87 for early and late passages.

Fig. 1, *A–D* shows the growth curves of 3T3R, SV101, and two calcium transformants, tx A-cl 1 and tx H, in 0.01 and 2.0 mM Ca^{++} media. An increased RGR was apparently due to an increase in the growth rate in low Ca^{++} medium, rather than to any inability of the calcium-transformed lines to grow in the normal amount of Ca^{++}. All of the cell lines tested were able to grow in media containing chelex-treated serum. The normal line, 3T3R, had a lower plating efficiency and doubling time in both 0.01 and 2.0 mM Ca^{++} than did the transformed lines. This did not affect the value of the relative growth rate since it was calculated as as ratio of the growth in 0.01 mM Ca^{++} relative to that in 2.0 mM Ca^{++}.

Transformed Phenotypes of Calcium-transformed Swiss 3T3 Clones

ANCHORAGE REQUIREMENT: Previous work has shown that not all SV40 transformants exhibit the same transformed phenotypes (9, 26). Therefore, the calcium transformants were tested in other transformation assays. The ability of a cell to grow without anchorage is the phenotype most strongly linked to tumorigenicity (31). All of the cal-

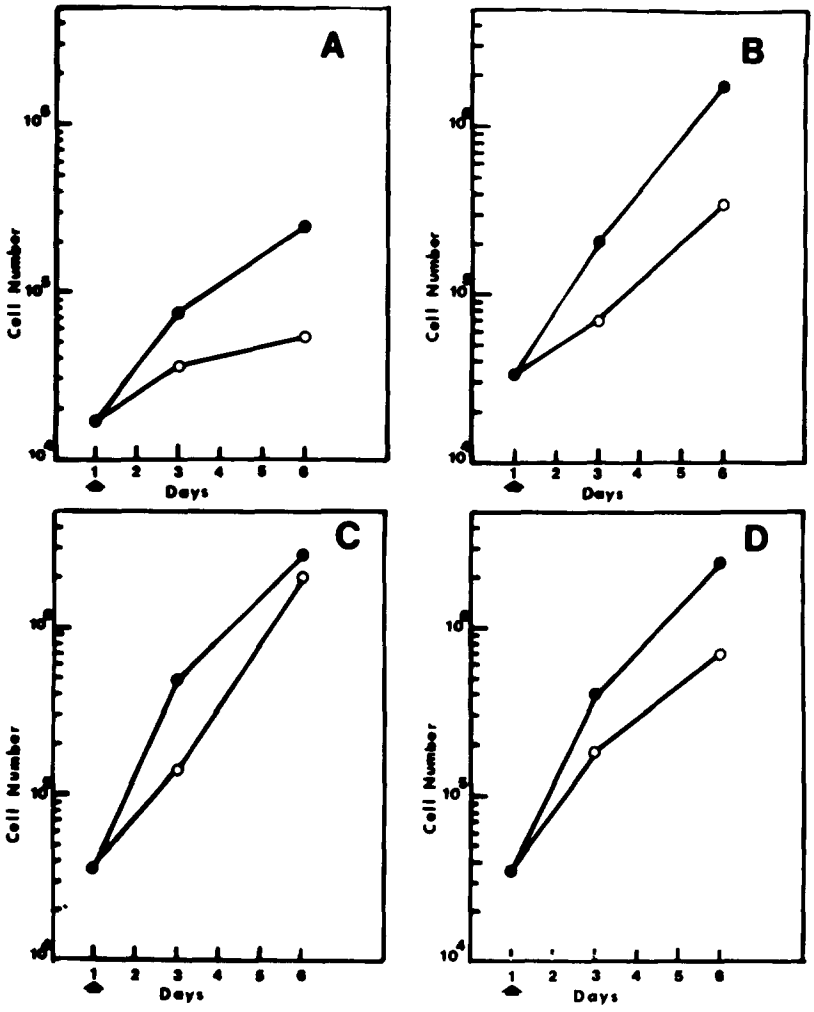

FIGURE 1 Growth of mouse cell lines in 0.01 or 2.0 mM Ca^{++}. ●, 2.0 mM Ca^{++}; O, 0.01 mM Ca^{++}. Cells were plated in regular growth medium, and the next day duplicate dishes were trypsinized and counted. Sister dishes were rinsed with 0.01 mM Ca^{++} medium and re-fed with either 0.01 or 2.0 mM Ca^{++} medium. Arrow indicates medium change. We counted duplicate dishes 3 and 6 d after plating. (*A*) 3T3R. (*B*) SV101. (*C*) tx A-cl 1. (*D*) tx H.

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cium-transformed clones were tested in at least two separate experiments for their growth in agarose. Table IV shows that all of the clones were anchorage-independent. There was a wide range in the ability to grow without anchorage from a very low plating efficiency (tx F, 0.033%) to a high response (tx I, 35.3%). It is interesting that tx F, which was T-negative (Fig. 2), had a low plating efficiency in agarose. The ability to grow without anchorage did not seem to be caused by the loss of the calcium requirement, because clones tx B, tx D, tx M, and tx N did not have reduced Ca^{++} requirements, yet grew in agarose with a wide range of plating efficiencies (Table IV).

VIRAL TUMOR ANTIGENS: All of the clones were examined for SV40 T-antigen by immunofluorescence. All of the clones except tx A and tx F were 100% T-positive when tested at the first passage after isolation (data not shown). When first tested, tx A was 84% T-positive, but upon subcloning tx A-cl 1 was 100% T-positive. Therefore we conclude that tx A was a mixed population of transformed and normal cells.

TABLE IV

Growth in Agarose of Calcium Transformants

Plating efficiency* (%)				
<0.001–0.009	0.01–0.09	0.1–0.9	1.0–9.0	10.0–99.0
3T3R	tx C	tx A	SV101	tx I
	tx D	tx B	tx J	tx L
	tx E	tx H	tx K	tx N
	tx F		tx M	
			tx O	

* Plating efficiency was measured as percent colonies in agarose > 0.2 mm per cell number inoculated.

Clone tx F was found to be T-negative both by immunofluorescence and by immunoprecipitation. By Southern blot analysis, tx F was found to contain integrated SV40 DNA, although it did not contain an intact *BglI–BamH1* early region fragment (Fig. 2*B*). A deletion or mutation at these restriction sites would result in the absence of an intact *Bgl–Bam* fragment and also could effect the coding region or control region of large T antigen leading to a lack of T antigen expression.

SV40 tumor antigens in these lines were also examined by immunoprecipitation. All of the clones except tx F contained the expected large and small T antigens at 94,000- and 17,000-mol-wt. All of the clones except tx D and tx F also contained the variant 100,000 mol-wt T (Fig. 2*A*). This super-T antigen has been shown to correlate with anchorage independence in a set of transformed and revertant cell lines (10). Clones tx D and tx F also have low plating efficiencies in agarose (Table IV), which further supports the association of the 100,000 mol-wt super-T antigen and anchorage-independence in the present study.

SERUM REQUIREMENT: Minimally transformed cells are able to grow in low concentrations of serum (25). In serum-free medium, the serum requirement seems to be linked to the requirement for insulinlike growth factor (Powers, R. S., S. Chen, and R. Pollack, submitted for publication). Table V shows that the RGR in 1% FCS of 3T3R was 0.36 and that of SV101 was 0.67. The RGRs of the isolated clones were clustered around that of SV101, including tx B, tx D, and tx M, which were not calcium-transformed. Clone tx C (which was calcium-transformed) was unusual in having a RGR in 1% FCS which was similar to that of 3T3R.

ACTIN CYTOSKELETON: SV40 transformants have been found to have disrupted actin cytoskeletons (24). The actin

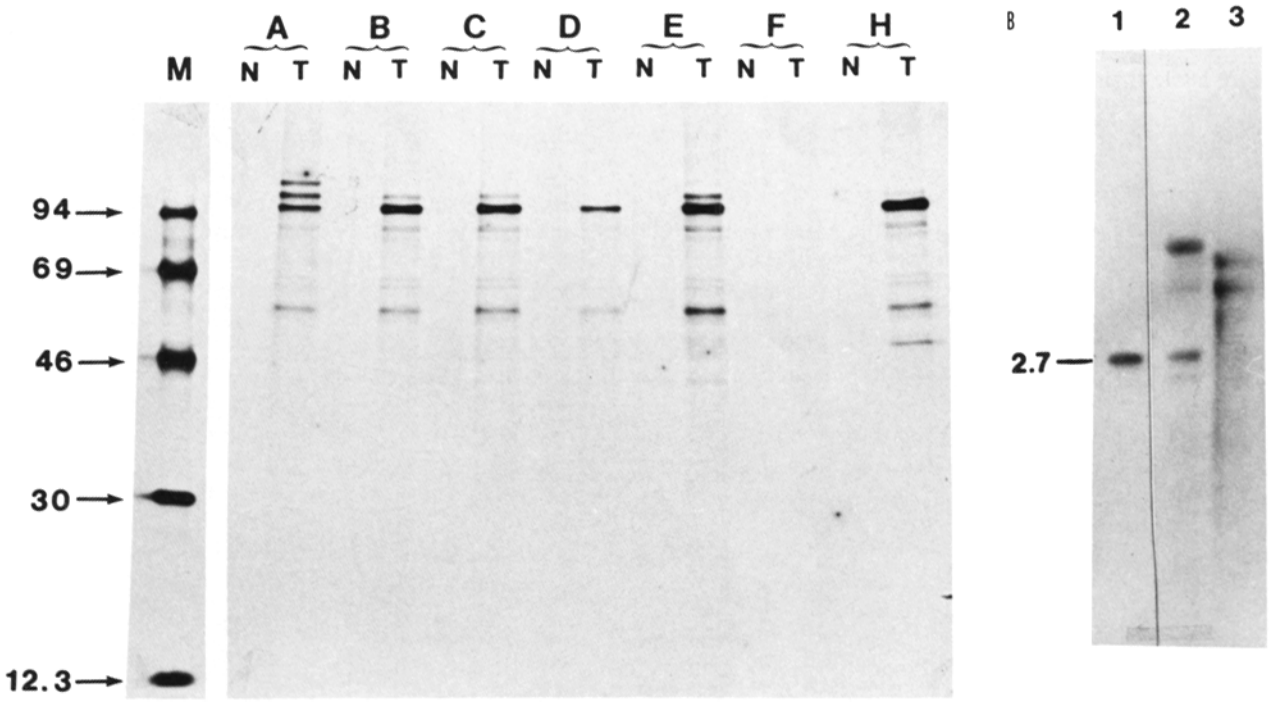

FIGURE 2 (*A*) Immunoprecipitation of viral T antigens in calcium-transformed mouse cell lines. Lane *M*, markers; lane *A*, tx A-cl 1; lane *B*, tx B; lane *C*, tx C; lane *D*, tx D; lane *E*, tx E; lane *F*, tx F; lane *H*, tx H. *N*, Normal serum; *T*, Tumor serum. (*B*) Southern blot of integrated SV40 DNA in calcium-transformed mouse cell lines. Lane *1*, *BglI* + *BamH1* digest of 10 pg SV40 DNA (~1 copy per haploid genome); lane *2*, tx A-cl 1; lane *3*, tx F. All lanes were probed with pSV Bgl–Bam plasmid that contained only the *BglI* to *Bam* early region fragment of SV40.

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TABLE V
Growth in 1% FCS of Calcium Transformants

RGR (1%/10%)*							
0.1–0.19	0.2–0.29	0.3–0.39	0.4–0.49	0.5–0.59	0.6–0.69	0.7–0.79	0.8–0.89
		3T3R	tx F	tx B	SV101	tx J	tx A
		tx C	tx N	tx D	tx H	tx O	tx I
				tx E	tx K		
					tx L		
					tx M		

* *RGR*, doublings in 1% FCS/doublings in 10% FCS.

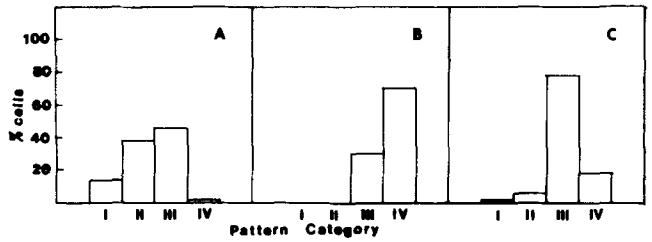

FIGURE 3 Actin patterns of 3T3R, SV101, and calcium transformants. Histograms of actin scores showing the average percentages of cells in each category. *Category I* cells have large cables filling the cytoplasm; *category II* cells have fine cables and a few large cables; *category III* cells have only fine cables; and *category IV* cells have no cables. (*A*) 3T3R; (*B*) SV101; (*C*) averages of all calcium transformants.

pattern of the calcium transformants was examined using rhodamine-conjugated phalloidin, and the cells were categorized into four classes (Fig. 3). Cells in class I are filled with large actin cables, cells in class II contain a few large actin cables, cells in class III contain only fine actin cables, and cells in class IV have no actin cables at all. All of the calcium transformants had reduced numbers of cells with large cables compared to 3T3R. Although the numbers of calcium-transformed cells with fine cables were lower than in 3T3R, they were not as low as in SV101. Thus large cables seem to be more labile than small cables. All of the clones tested had a disrupted actin cytoskeleton, although not all had a reduced Ca^{++} requirement.

DISCUSSION

We conclude that clones selected from low-Ca^{++} medium are "fully transformed" by all current established criteria. In addition, some but not all of these clones have a stably reduced Ca^{++} requirement. Apparently SV40 transformation does not always permanently reduce the Ca^{++} requirement, although each clone must have had a transiently reduced Ca^{++} requirement in order to have formed a dense focus. All of the clones have lost the anchorage requirement; all but one have lost the serum requirement; all but one contain viral T antigens; and all have disrupted actin cytoskeletons.

Although the SV40 transformant, SV101, is fully transformed by other traditional criteria, it is not transformed in the low-Ca^{++} growth assay. The revertant lines that have regained either the serum requirement, the anchorage requirement, or both also do not grow in low Ca^{++}. Therefore, the ability to grow in 0.01 mM Ca^{++} is not the direct consequence of the acquisition of either serum or anchorage transformation.

Selection in low-Ca^{++} medium after SV40 infection of Swiss 3T3 cells can produce transformants that are independent of reduced Ca^{++}. The transformants we have selected in reduced Ca^{++} medium were dense foci against a background of flat cells. Thus we also selected for cells that were able to grow to high density. Inasmuch as no dense foci were observed in mock-infected cells in 0.01 mM Ca^{++}, the ability to grow in reduced Ca^{++} was caused by SV40 infection. This ability was not due to selection of normal 3T3 cells in low Ca^{++}, because monolayers of mock-infected cells tested after a 3-wk growth in 0.01 mM Ca^{++} did not show a decreased calcium requirement. Other investigators have found a decreased Ca^{++} requirement in cells transformed by chemicals or viruses without selecting for this phenotype (1, 3, 5, 16). However, Paul and Ristow (23) also found that Swiss 3T3 doubled once or twice in 0.01–0.03 mM Ca^{++} and that the RGR of unselected SV40-transformed Swiss 3T3 and benzo[a]pyrene-transformed 3T3 was only ~0.5 in 0.01 mM Ca^{++} relative to 1.8 mM Ca^{++}.

The physiological basis for the Ca^{++} requirement remains to be elucidated. It is known that Ca^{++} is involved in the proliferative response to hormonal stimulation and is involved in cAMP and calmodulin regulation of cellular processes (12). There seems to be a redistribution of the Ca^{++} upon serum stimulation, although there is no change in the total Ca^{++} concentration (14, 17, 35). It has also been shown that normal mouse cells arrest at the G_1 phase of the cell cycle in medium depleted of Ca^{++} and will proceed when Ca^{++} is added (5, 23). This block can be overcome by TPA (12-O-tetradecanoyl-phorbol-13 acetate) treatment possibly by increasing the membrane permeability to Ca^{++} (4).

Some transformed cells have been found to have increased levels of calmodulin (8). Increases in the calmodulin concentration have also been observed in a variety of untransformed cells during late G_1 and early S phase of the cell cycle, the calcium-sensitive point of the cell cycle of normal, but not of transformed cells (13). However, Durkin et al. (13) found that the increased calmodulin concentration is not responsible for the reduced serum requirement of transformed rat cells.

Viral T antigens are found in all but one of the calcium transformants after SV40 infection. By immunoprecipitation the viral protein content of most of the calcium-transformants is similar to that of other SV40 transformants. It has been suggested that 94,000-mol-wt T antigen is necessary for the establishment and maintenance of transformation (for a review, see reference 34), while the role of 17,000-mol-wt T antigen in transformation is less well understood. It would be interesting to test viral mutants and fragments of SV40 DNA to determine which viral proteins, if any, are necessary for calcium transformation.

SV40 transformation of established cells results in a wide variety of phenotypes. The SV40 proteins expressed in mouse cells are limited in number, yet can elicit a pleiotropic response. This could be due to the interaction of SV40 DNA or proteins with various cellular genes. Scott et al. (30) found that SV40 infection of mouse cells activated several sets of cellular genes. One of these sets required either a functional

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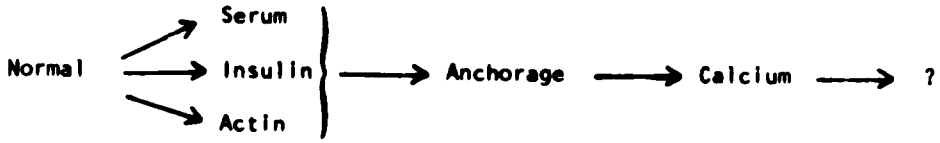

FIGURE 4 Proposed scheme of transformation progression. In this model, transformation progresses from normal cells to minimal transformation, expressing only the loss of the serum requirement, to the fully transformed phenotype with the addition of each characteristic.

T antigen or other transforming agents such as retroviruses or chemical carcinogens for its activation. SV40 DNA or proteins may also interact with cellular oncogenes (18). It has been suggested recently by Weinberg and colleagues (19) that two complementary genes are needed to transform primary rat cells (29), while only one is needed for established cell lines. Swiss 3T3 cells are already established. An additional function is provided by SV40 which leads to transformation.

Clone tx F is a "full transformant," including the phenotype of calcium independence, yet it does not express T antigen. As shown by Southern blot analysis, this clone contains integrated SV40 DNA, but lacks an intact early region as defined by restriction enzyme sites. It is possible that tx F contains a mutant SV40 molecule, which results in a lack of T antigen and in an off-size early region fragment. It is also possible that tx F is a spontaneous transformant and that the integrated SV40 DNA is incidental to the high multiplicity of infection. However, the fact that we did not observe any dense foci in the mock plates suggests that tx F is probably not a spontaneous transformant. Other T-negative transformants have been observed, but these were minimal transformants, expressing only the phenotype of growth in low serum (25). Therefore, it is possible that in the case of tx F the SV40 DNA acted as a cellular mutagen and activated cellular gene(s) that allowed for transformation. It would be interesting to see if selection in low Ca^{++} somehow drives the cells to the fully transformed state without a fully functional SV40 T antigen by studying a series of T-negative clones selected in this system.

Ca^{++} and insulin may act coordinately to control the growth of the cell. In normal cells both Ca^{++} and insulin are required for division. SV40-transformed 3T3 cells can be selected to grow without insulin in serum-free medium (Powers, R. S., S. Chen, and R. Pollack, submitted for publication). These insulin transformants in the presence of fetal calf serum lost the Ca^{++} requirement (Nicholson, N., unpublished observations). It would be interesting to determine whether Ca^{++} is required in insulin-free serum-free medium for the growth of insulin-transformants, and whether insulin is necessary for the growth of calcium-transformants in Ca^{++}-free serum-free medium.

An ordered array of phenotypes has been shown in SV40 transformants, ranging from only serum transformed to anchorage transformed (27). Anchorage transformants are always serum-transformed, but serum transformants are sometimes anchorage-dependent. Clones with decreased serum requirements and insulin requirements and with altered cytoskeletons may or may not have lost the anchorage requirement, but anchorage-independent clones are invariably transformed in the other assays. The clones described in this paper that were selected in low Ca^{++} were all but one transformed in the serum assay, all transformed in the anchorage and actin assays, and 10/14 of them were stably calcium-transformed. This suggests that in the ordering of transformed phenotypes the loss of the calcium requirement is the most fully transformed (Fig. 4). It remains to be seen what other transformed phenotypes may be revealed if new selective pressures are applied.

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