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Transformation of human skeletal muscle cells by simian virus 40

(differentiation/myoblast fusion/creatine kinase/viral DNA integration/tumor antigen)

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ABSTRACT **Molecular studies of the biochemical alterations involved in human myopathies have been restricted because of the finite life-span and slow growth rate of cultures derived from primary tissue. Because the tumor virus simian virus 40 (SV40) can alter both the growth properties and longevity of human cells, we have infected skeletal muscle cultures derived from four biopsies with a small-plaque variant of SV40 and analyzed the biological and biochemical properties of cloned myoblast derivatives. At early times after infection, myoblasts fused normally into multinucleated myotubes, and both unfused and fused cells contained SV40 tumor antigen (T antigen). After six to eight subcultures after infection, the ability of myoblasts to fuse diminished, and clonal cell lines were generated with increased growth rates and saturation densities. Transformed cultures also lost contact inhibition of growth and became anchorage independent. Unlike untransformed myoblasts, SV40-transformed clones did not undergo an increase in creatine kinase activity or a transition of creatine kinase isoenzymes from the BB form to the muscle-specific MM form. Analysis of the pattern of SV40 DNA integration by Southern blotting hybridization analysis in two cloned SV40-transformed myoblast cell lines (KJ-SV40 and PK-SV40) indicated that KJ-SV40 contained at least one site of SV40 DNA integration into chromosomal DNA and PK-SV40 contained at least three sites of SV40 DNA covalently linked to cellular DNA. Cell lysates and growth medium from PK-SV40 transformants contained infectious small-plaque variant SV40, whereas KJ-SV40 did not contain or produce detectable virus. These studies demonstrate that human myoblasts can be immortalized by SV40. This procedure may prove useful for generating large quantities of genetically deficient human cells for biochemical and molecular analysis.**

Recent improvements in cell culture techniques now permit the *in vitro* growth of both normal and genetically deficient human skeletal muscle (1–5). Under appropriate conditions, satellite cells derived from human skeletal muscle biopsies can develop into cultures that recapitulate ontogenic myogenesis as normally observed in regenerating muscle *in vivo* (6–8). Myogenic stem cells fuse to form multinucleated syncytia (myotubes) that no longer undergo conventional DNA synthesis or cell division. In addition, these myotubes produce large quantities of muscle-specific contractile proteins (4, 9) and sarcoplasmic enzymes (3–5) and develop muscle-specific membrane markers, such as acetylcholine receptors (4). The utility of primary cultures for analyzing myopathies is often difficult, however, because of the limited growth potential of these cultures. Fogel and Defendi (10) have demonstrated that fetal myoblasts from humans and rodents are susceptible to infection by the DNA tumor virus simian virus 40 (SV40) and polyoma, whereas myotubes are resistant to infection. Infected myoblasts were fusion competent and formed myotubes even though they contained tumor antigen (T antigen). In the present study, we demonstrate that SV40 can stably transform human myoblasts into immortal cell lines that, however, do not undergo terminal differentiation into myotubes.

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MATERIALS AND METHODS

Muscle Cultures. Cultures were grown from four skeletal muscle biopsies obtained for diagnostic evaluation, with advised consent, which by established criteria were found to be free of muscle disease. PK-SV40 was derived from muscle of a 2-yr-old girl, and KJ-SV40 was derived from a 30-yr-old man. The cultures were first grown as explants in 35-mm Costar (Cambridge, MA) cluster dishes and then as monolayers in 250-ml culture flasks (Falcon; Becton Dickinson Labware, Oxnard, CA), after preplating for 15 min to remove fibroblasts (3, 11). The standard medium was Eagle's minimal essential medium enriched with vitamins, nonessential amino acids, and pyruvate (GIBCO) to which 15% pretested fetal bovine serum, penicillin (75 units/ml), and streptomycin (75 μg/ml) were added. To promote myoblast fusion, the enrichments were eliminated from the medium and the fetal bovine serum was reduced to 8%. Details of culture methods have been described (3).

Virus. Small-plaque SV40 (strain 776) (12) was applied to prefused, subconfluent muscle cultures at 50 or 100 plaque-forming units per cell in standard medium containing 1% heat-inactivated fetal bovine serum (30 min at 56°C) for 18–24 hr. The cultures were then rinsed with Earle's balanced salt solution, trypsinized, and seeded in 100-mm Falcon Petri dishes (200 or 400 cells per dish) and left undisturbed for 2 wk in a 5% CO_2 incubator kept at saturation humidity. Muscle cell clones were isolated with glass cloning rings, attached with sterile silicone grease, trypsinized, and transferred to 35-mm dishes for further growth in standard medium. Only clones showing a few myotubes in the center of the cell colony were isolated. Cultures were routinely split 1:3 as the cell sheet approached confluency, prior to myoblast fusion.

T Antigen. Fluorescein-tagged anti-SV40-T-antigen polyclonal antibodies (Flow Laboratories) diluted 1:4 in phosphate-buffered saline (pH 7.4) were applied for 45 min to cultures grown on 22-mm coverslips, after a rinsing with Earle's balanced salt solution and a 10-sec fixation in acetone at 4°C. The coverslips were then rinsed five times for 5 min in phosphate-buffered saline, mounted in phosphate-buffered saline/glycerin, 2:8 (vol/vol), and examined and photographed in a Leitz fluorescent microscope with epiillumination using a BG12 exciter filter and an OG barrier filter.

Mitotic Index. The mitotic index was determined in cultures grown on coverslips, fixed with 100% ethanol for 10 min, rinsed with distilled water, and stained with Giemsa solution, 1:10 (vol/

Abbreviations: SV40, simian virus 40; T antigen, tumor antigen; CK, creatine kinase; CK-BB, CK-MM, and CK-MB, isoenzymes of CK.

vol) in water for 15 min. At least 1,000 cells per random field were counted for each preparation with an ocular grid at ×40 magnification.

Fusion Index. Myoblast fusion was quantitated by determining the percentage of total cell nuclei in myotubes in Giemsa-stained cultures as described for mitotic indices.

Growth Curves. For growth curves, cultures were grown in 35-mm dishes (four cultures per time point) seeded at 5×10^4 cells per dish in 2 ml of standard medium and were fed 48 hr and 144 hr after planting.

Creatine Kinase (CK). CK determinations were carried out on supernates of cell homogenates disrupted by ultrasonication and centrifuged at 10,000 × *g* for 15 min at 4°C. Total CK activity was determined by a modification of the method of Oliver (13) as described (3, 14). Total protein was measured as described by Lowry *et al.* (15). CK isoenzymes were separated electrophoretically in a Helena apparatus with Titan III cellulose acetate membranes (Helena Laboratories, Beaumont, TX) presoaked in 0.05 M Tris/barbital buffer, pH 8.6 (14). The air-dried membranes were photographed under UV light (360 nm) with a Polaroid camera, Polaroid 107 film, and a 2C filter.

Assay for SV40 Infections. Cell lysates from freeze/thawed transformed cells and conditioned growth medium (5 days) from transformed cells were analyzed for SV40 by plaque assay on CV-1 monkey cells (16). As a positive control, wild-type SV40 was titrated in parallel with transformed cell lysates and conditioned medium on CV-1 cells.

Analysis of SV40 DNA Integration in Chromosomal DNA of Transformed Muscle Cells. The pattern of SV40 DNA integration in the PK-SV40 and KJ-SV40 transformed muscle clones was determined by Southern blotting hybridization analysis (17). Briefly, 10 μg of high molecular weight cellular DNA was digested with an excess of the appropriate restriction endonucleases, and the resulting DNA fragments were separated by electrophoresis on a 0.8% agarose slab gel and transferred to nitrocellulose filters, as described (18). Wild-type SV40 form I DNA was labeled *in vitro* by nick-translation (19) and was hybridized to cellular DNA to identify the presence of viral-specific sequences. After hybridization, the filters were washed, dried, and autoradiographed on Cronex 2^B x-ray film.

RESULTS

Immunofluorescence Analysis of SV40 T-antigen Synthesis in Infected Human Myoblasts. Subconfluent cultures tested 2 wk after virus application had 4–5% T-antigen-positive cells. Nuclear fluorescence was observed in both mononuclear myoblasts and developing myotubes after staining with fluorescein-tagged SV40 T-antigen antibody (Fig. 1). When T-antigen-positive cell cultures were passaged eight times (9 wk after application of the virus), only 5% of the nuclei were present in myotubes at confluency. At this stage the cultures showed ≈80% T-antigen-positive nuclei. In contrast, parallel untransformed parental cultures exhibited an ≈78% fusion index when propagated under similar culture conditions as their SV40-transformed counterparts. With repeated passage, nonfusing cell populations developed that were 100% positive for T antigen.

Cell Morphology and Growth Properties of SV40-Transformed Human Myoblasts. Untransformed human muscle cultures consisted predominantly of triangular (sometimes multipolar) mononuclear cells that acquired a fusiform morphology as the cells reached confluency and lined up in preparation for fusion. During the first 3 wk after SV40 infection, the morphology and growth properties of virally infected and uninfected myoblasts were similar. After further subcultivation of myogenic subclones, however, distinct foci of tightly packed, flattened multipolar cells with enhanced mitotic indexes developed. At this stage of transformation, the fusion index was never more than 6% and the myotubes developed perinuclear vacuoles. The ability of cloned SV40-transformed myoblasts to spontaneously form myotubes was totally eliminated after continued propagation. Subclones of the SV40-transformed cells, prepared after 14 wk in culture never showed multinucleated myotubes. Furthermore, reduction of the serum concentration of the medium to 8% promoted myotube formation in the untransformed control cells but had no effect on PK-SV40 or KJ-SV40 cells. Of the four cloned SV40-transformed myoblast cell lines isolated from different skeletal muscle biopsies, only PK-SV40 exhibited indications of a "crisis" (20, 21) period (i.e., reduction in growth rate, sloughing off of moribund cells with pycnotic nuclei, inability to be subcultured, etc.) after 10–12 serial passages. With further subculture, however, PK-SV40 recovered from this transitional crisis period, and the cells displayed properties similar to those of the three other SV40-transformed cell lines, which so far never showed obvious signs of crisis. All four SV40-transformed clones exhibited enhanced growth rates in comparison with control cultures and continued to increase in cell number after reaching confluency, whereas untransformed myoblasts reached a lower saturation density at confluency, when the first myotubes became apparent (Fig. 2). When DNA synthesis was analyzed in subconfluent cultures by

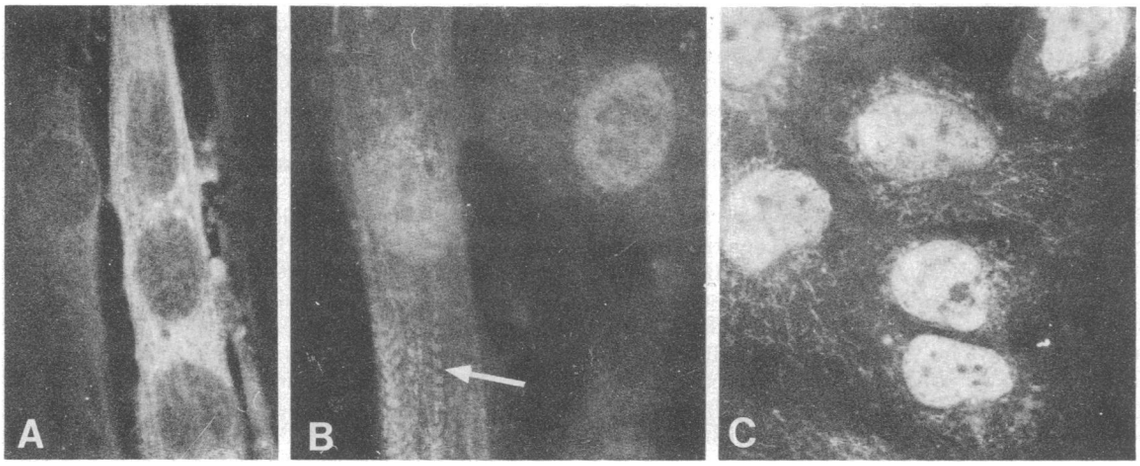

FIG. 1. Identification of SV40 T antigen in nuclei of virally infected cells. (*A*) Uninfected control culture. Nuclei are unstained; cytoplasmic staining is nonspecific, not due to SV40 T antigen. (*B*) Myogenic clonal culture showing T-antigen-positive nuclei at early stages of transformation; nonspecific cytoplasmic staining reveals cross-striations in the myotube (arrow). (*C*) T-antigen-positive SV40-transformed myoblasts in subculture, 6 months after virus application; cells no longer fuse.

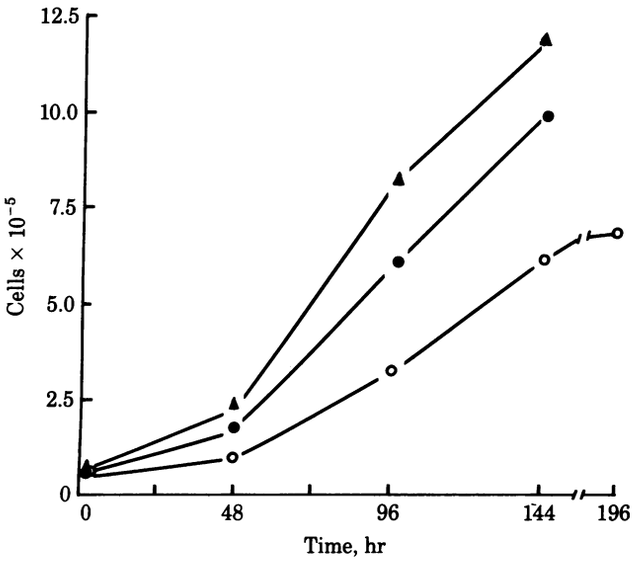

FIG. 2. Growth curves of uninfected and SV40-transformed clonal cultures. ○, Control culture; ▲, PK-SV40; ●, KJ-SV40.

[^{3}H]thymidine labeling for 18 hr and autoradiography, 28–37% of cell nuclei in untransformed cultures, but 95–98% of the nuclei in SV40-transformed cultures, were densely labeled.

SV40-transformed myoblasts did not adhere to the plastic substratum as tenaciously as did normal myoblasts and detached within 2 min after exposure to 0.125% trypsin, as opposed to 5 min for normal controls. When assayed after ≈20 passages following virus infection for anchorage independence [i.e., the ability to form colonies when suspended in 0.4% agar (22, 23)], both PK-SV40 and KJ-SV40 formed colonies with ≈0.1% and ≈0.3% efficiency, respectively. As had been observed with other SV40-transformed cell lines (24), both PK-SV40 and KJ-SV40 tested after 25 passages did not form tumors in athymic *nude* mice. PK-SV40 (passage 38) and KJ-SV40 (passage 33) grown in medium supplemented with 1% fetal bovine serum had doubling times of 42 hr and 48 hr, respectively.

CK Isoenzymes in SV40-Transformed Myoblasts. Total CK activity of normal, untransformed human muscle cultures increases 2-fold or more immediately after optimal myoblast fusion (14). Total CK activities in PK-SV40 and KJ-SV40 cells never showed such increases: homogenates of untransformed myoblasts and myotube cultures (48 hr after fusion) had total CK activities of 0.094 ± 0.018 and 0.221 ± 0.011 unit, respectively. PK-SV40 and KJ-SV40 never had CK activities higher than 0.104 ± 0.013 unit, even after cultivation of up to 2 wk.

In unfused myoblasts the BB isoenzyme form of the enzyme CK (CK-BB) was found (Fig. 3). During the process of myoblast fusion in *in vitro* cell culture systems and *in vivo*, there is an increase in CK activity and a gradual replacement of CK-BB with a muscle-specific CK isoenzyme (CK-MM), which is under separate genetic control (see refs. 3 and 8). Because CK-B and CK-M subunits can hybridize randomly under physiological conditions, a third CK isoenzyme (CK-MB) migrating intermediately between CK-MM and CK-BB also was detected during myoblast fusion. SV40-transformed myoblasts not only lost their ability to fuse but also failed to show isoenzyme transition from CK-BB to CK-MM (Fig. 3). Even prolonged maintenance (up to 2 wk) of SV40-transformed cells at confluency did not result in fusion or CK isoenzyme changes.

Viral DNA Integration in SV40-Transformed Myoblast Clones. Southern blotting hybridization analysis (17) was used to examine the pattern of SV40 DNA integration in the cellular

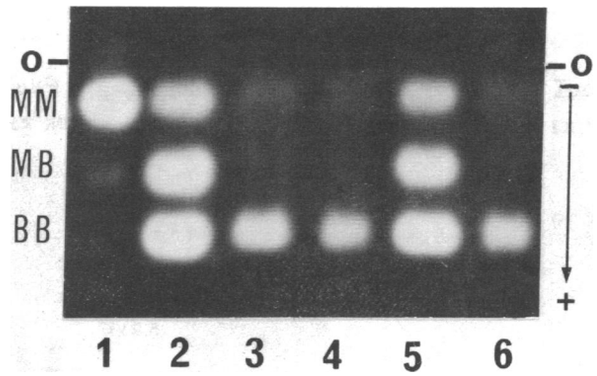

FIG. 3. CK isoenzyme activities revealed by cellulose acetate electrophoresis. Lanes: 1, intact mature muscle; 2 and 5, untransformed control muscle cultures after fusion; 3 and 4, PK-SV40 and KJ-SV40 clonal cultures; 6, untransformed control muscle culture before fusion.

genomes of PK-SV40 and KJ-SV40 (Fig. 4*A*). The number of unique SV40 integration sites in the transformed myoblasts was determined by cleaving cellular DNA with the restriction endonuclease *Sac* I or *Xba* I and hybridizing the separated DNA fragments with a ^{32}P-labeled nick-translated SV40 form I DNA probe. These enzymes were chosen because they do not cleave sequences within SV40 DNA (Fig. 4*B*). Both cell lines contain integrated SV40 DNA because the molecular weights of the hybridized molecules were heavier (migrated slower in agarose gels) than did SV40 form I marker DNA (Fig. 4*A*, lane M). In the case of PK-SV40, three bands were generated when cellular DNA was cleaved with *Sac* I, whereas only two bands were produced with *Xba* I (Fig. 4*A*). Because both enzymes do not cleave at any site within the SV40 genome, there are three alternative explanations for the development of three bands with *Sac* I and only two bands with *Xba* I in the PK-SV40 cell line. First, the two bands present in the *Xba* I digest could represent three bands that are poorly resolved because of similar molecular weights. Second, tandem repeats of SV40 DNA might have occurred and, in the process, resulted in deletions. Third, although PK-SV40 is of clonal origin, because this line releases infectious virus and also may undergo chromosomal changes with passage, there is the possibility that the sites of integration may change in a proportion of the cells during subsequent cultivation. Three additional faint bands are present also in the *Xba* I digests of KJ-SV40, which also may be a reflection of clonal instability.

To define more precisely the locations of the integrated SV40 sequences within the transformed cells, cellular DNA was cleaved with *Bam*HI or *Eco*RI, each of which cleaves SV40 DNA one time (Fig. 4*B*). If tandem SV40 DNAs were present, one would predict at least three viral specific bands to be present, and one of the bands would correspond in size to form III SV40 DNA. When the DNA of PK-SV40 was digested with *Bam*HI or *Eco*RI, at least six prominent bands were present on autoradiographs, which would be predicted because at least three sites of viral DNA integration exist. Because no labeled molecules comigrated with SV40 form III DNA, and if no gross deletions in the SV40 genomes occurred, it can be assumed that no tandem duplication of viral sequences exist in the PK-SV40 cell line. When the DNA of KJ-SV40 was digested with *Bam*HI or *Eco*RI, two viral-specific bands were present, which indicates one major site of viral DNA integration and no tandem integration.

To determine more precisely which SV40 DNA sequences are present in PK-SV40 and KJ-SV40, cellular DNA was cleaved with *Pvu* II or *Hpa* I and hybridized with marker (Fig. 4*A*, lanes M) SV40 DNA cleaved with the same enzyme. This analysis (Fig. 4*A*) indicates the increased complexity of the PK-SV40

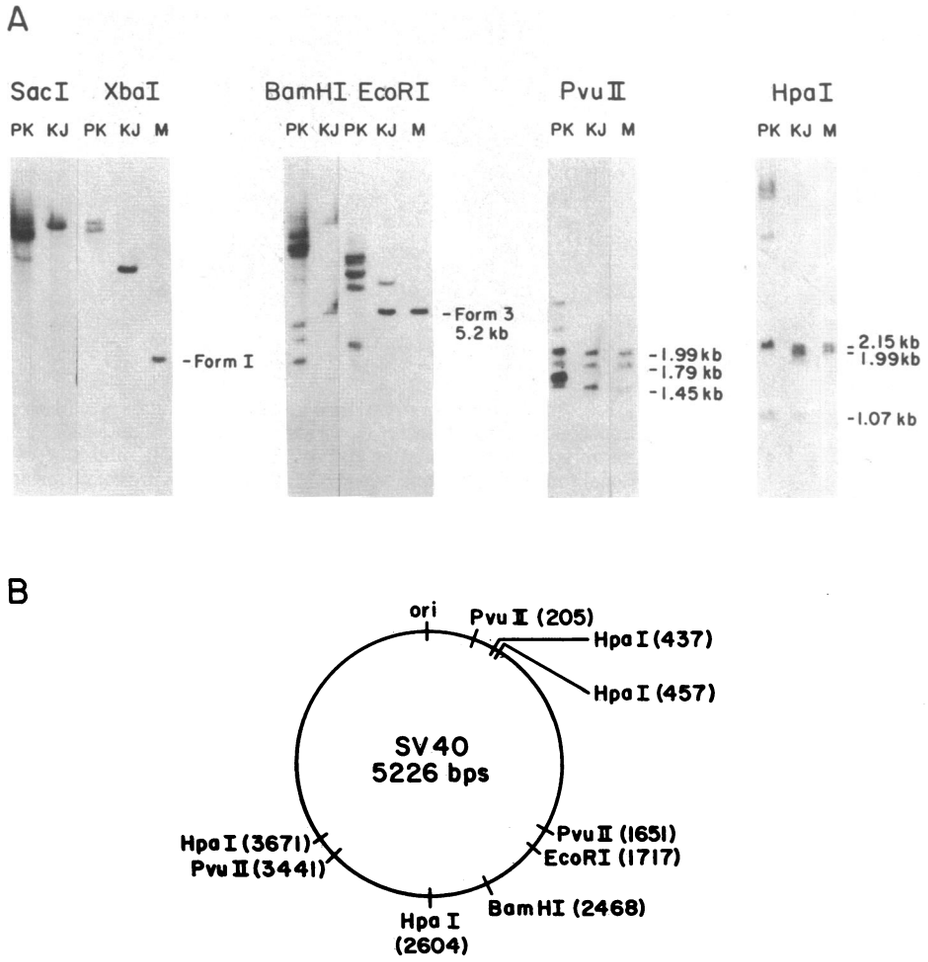

FIG. 4. SV40 DNA integration in PK-SV40 and KJ-SV40, as revealed by Southern blotting hybridization analysis (17). (*A*) Cellular DNA cleaved with *Sac* I, *Xba* I, *Pvu* II, *Bam*HI, *Eco*RI, or *Hpa* I. M refers to the appropriate marker SV40 DNA, cleaved with the same restriction endonuclease as cellular DNA. (*B*) Schematic representation of SV40 DNA indicating the site(s) of cleavage with the restriction endonuclease *Bam*HI, *Eco*RI, *Pvu* II, or *Hpa* I.

versus the KJ-SV40 transformed clones. Further studies with specific nick-translated SV40 DNA fragments will be necessary to directly identify which hybridized regions correspond to which viral DNA sequences in the PK-SV40 transformants.

Production of SV40 by Transformed Myoblast Clones. Transformation of human cells by SV40 may or may not result in cultures producing infectious virus (21, 25, 26). Therefore, we have determined if the supernatant or cell lysates from PK-SV40 and KJ-SV40 contain infectious SV40. As can be seen in Table 1, both the culture medium and cell lysates of PK-SV40 contained infectious virus, whereas KJ-SV40 did not contain detectable SV40. As would be predicted, the strain of SV40 produced by PK-SV40 was the small-plaque variant of SV40 (12) that was used to transform these cells.

Table 1. Production of SV40 by transformed human myoblasts

Virus or transformed cell line*	Dilution	Plaques per dish†	Titer, pfu/ml
Wild-type SV40	10^{-6}	7.5	7.5×10^7
PK-SV40 cell lysate	10^{-4}	400	2×10^7
supernate	10^{-6}	170	8.5×10^6
KJ-SV40 cell lysate	10^0	0	0
supernate	10^0	0	0

pfu, Plaque-forming units.

* Cell lysates were obtained from transformed cells by three cycles of freeze/thawing followed by low-speed centrifugation to remove cellular debris. To test for virus release by transformed myoblasts, growth medium from semiconfluent cultures was collected after 5 days of growth and assayed on CV-1 cells. Wild-type SV40 was supplied by Dr. P. Tegtmeyer (State Univ. of New York at Stony Brook, Stony Brook, NY) (27).

† Values presented represent the average from six plates for each. Serial dilution replicates varied by 15%.

DISCUSSION

A useful strategy for prolonging the life-span of normal human cells in culture is transformation with the DNA tumor virus SV40 (21). Unlike monkey cells, which are permissive for SV40 infection (i.e., the virus replicates, producing large quantities of infectious virus and cell death), human cells can be stably transformed by SV40 resulting in cultures that either continue to produce infectious virus or do not contain detectable SV40 in cell lysates or culture medium (21). In the present study we have demonstrated that transformation of human myoblasts, derived from mature muscle with SV40 results in both producer (PK-SV40) and nonproducer (KJ-SV40) transformants (Table 1). Analysis of the pattern of SV40 DNA integration in PK-SV40 and KJ-SV40 indicate that the site and number of viral DNA inserts in these clones are different (Fig. 4). Although the mechanism involved in continued virus production after SV40 transformation of human cells is not known, virus production is most likely a reflection of the molecular interactions between the cellular and integrated viral genomes and is not simply a function of the type of target cell being transformed.

Human cells infected with SV40 often exhibit a crisis phase characterized by a reduction in proliferative capacity, abnormal mitoses, cell detachment, and death (20, 21). Cells that survive this crisis period usually exhibit a set of phenotypic changes associated with transformation, including an apparently unlimited life-span. A crisis phase is not an absolute criterion for suc-

cessful SV40 transformation of human cells because SV40-transformed thyroid cells (28), brain cells (29), and keratinocytes (30–32) have developed into established cell lines without displaying alterations in proliferative capacity. Similarly, of the four SV40-transformed myoblast cell lines studied, only PK-SV40 displayed indications of a crisis period between passages 10 and 12. The three clones that did not show signs of an obvious crisis have been passaged for 4 (two clones) or 8 months (one clone) without alterations in monolayer growth properties. It is conceivable that a crisis period may occur after more prolonged periods of cultivation. It appears unlikely, however, that the absence of a crisis period is cell-type specific.

Transformation of specialized human cells by SV40 has resulted in (*i*) the continued expression of specialized functions in differentiated cells (33), (*ii*) a reduction in the expression of differentiated functions (34), or (*iii*) a complete elimination of specialized functions (31). In the case of SV40-transformed adult prostate cells, high levels of acid phosphatase were still observed after transformation (35). Similarly, SV40-transformed human parathyroid adenoma cells continued to produce parathyroid hormone through 9.5 months in culture (33). When human vascular endothelial cells were transformed by SV40, they continued to produce angiotensin-converting enzyme but did not produce factor VIII antigen and no longer showed endothelial-specific organelles (34). Transformation of human keratinocytes by SV40 has been shown to impair normal differentiation (30–32). With repeated passage, SV40-transformed keratinocytes were incapable of differentiating into squames, a property of untransformed differentiating keratinocytes (31). Similarly, SV40-infected myoblasts differentiated normally for more than 3 wk after addition of the virus, but with further subculturing the ability of T-antigen-positive transformed myoblasts to differentiate was reduced and ultimately eliminated.

The SV40-transformed myoblast clones described in this paper exhibit a number properties associated with transformation—i.e., enhanced growth rate, increased saturation density, anchorage independence, and unlimited growth potential. However, SV40-transformed myoblasts do not fuse into multinucleated myotubes and do not show increases in CK activity or isoenzyme transitions from CK-BB to CK-MM. Even though these SV40-transformed muscle cell lines do not differentiate normally, transformation with SV40 may be useful for obtaining large quantities of genetically deficient cells from affected individuals. This approach seems feasible because SV40-transformed cell lines derived from genetically abnormal skin fibroblasts have been shown to produce the same abnormal gene products that are present in the untransformed cells from which they were derived (36). In addition, studies by Chou (37) have indicated that human placental cells can be transformed by temperature-sensitive mutants of SV40 (*tsA*). *tsA*-transformed placental cells grown at 40°C display a normal phenotype and express differentiated functions, including high levels of alkaline phosphatase and elevated levels of human chorionic gonadotropin. When grown at 33°C, *tsA*-transformed placental cells exhibit a transformed phenotype and reduced levels of the differentiated gene products. A similar situation has been observed in human epidermal cells, transformed by transfection with DNA from a *tsA* mutant of SV40 (32): the presence of keratin and crosslinked envelopes was decreased when transformants were cultured at the permissive temperature (33°C). These differentiating functions could be restored, however, when transformed cells were incubated at the nonpermissive temperature (40°C). Our present studies indicate that a variant of wild-type SV40 can stably transform human myoblasts. Temperature-sensitive SV40-transformed myoblasts from normal and genetically diseased individuals should prove invaluable for analyzing the molecular basis of specific human myopathies caused by abnormal muscle-specific gene products and the molecular events involved in muscle cell differentiation.

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