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Expression of simian virus 40 large T (tumor) oncogene in mouse chondrocytes induces cell proliferation without loss of the differentiated phenotype

(differentiation/retrovirus)

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ABSTRACT **We have infected primary embryonic mouse limb chondrocytes with a retrovirus carrying simian virus 40 early regions and have obtained a monoclonal mouse chondrocyte line, MC615, that was able to grow on culture dishes for at least 7 months and 20 passages. MC615 cells show expression of simian virus 40 large T (tumor) antigen and express markers characteristic of cartilage *in vivo*, such as types II, IX, and XI collagen, as well as cartilage aggrecan and link protein. These data show that cell growth induced by large T oncogene expression does not prevent the maintenance of the chondrocytic phenotype.**

The first step in development of long bones is the differentiation of chondrocytes from mesenchymal cells. The chondrocytes produce an extracellular matrix (1) consisting of complexes of cartilage aggrecan, hyaluronan, and link protein embedded within a framework of collagen fibrils (2–4). The fibrils are composed of fibrillar collagens (mostly type II with smaller amounts of type XI) and are decorated on their surface with the nonfibrillar collagen type IX (5, 6). During endochondral ossification, the chondrocytes go through a process of maturation, characterized by onset of expression of the short-chain collagen type X (7), loss of aggrecan and type II collagen expression (8), and cellular hypertrophy.

Cloning of genes that control chondrocyte differentiation has not been possible because chondrocytes are unstable in culture (9–11), and stable cell lines have been difficult to generate. The *myc* oncogene has been used to stabilize avian (12–14) and rat chondrocytes (15), but this usually leads to a depression of type II collagen expression. Also, cell lines isolated from human chondrosarcomas (16) exhibit loss of proliferative capacity during subculture and extensive chromosomal abnormalities.

We report here the establishment of a monoclonal mouse chondrocyte cell line, termed MC615, after infection with a recombinant retrovirus transducing the large T (tumor) antigen of simian virus 40 (SV40) (17). MC615 cells show a polygonal, chondrocyte-like morphology and form nodules that stain with Alcian blue. In addition, they express types II, IX, and XI collagen as well as cartilage aggrecan and link protein.

MATERIAL AND METHODS

Primary Cell Culture. Chondrocytes were isolated from fore- and hindlimbs of 14-day-old mouse embryos (CD-1 strain; Charles River Breeding Laboratories). Treatment of whole limbs with 0.05% trypsin (GIBCO) for 1 hr removed the peripheral tissues; a second digestion of the core regions with 0.25% trypsin and 0.1% bacterial collagenase type I (Sigma) for 2 hr followed by gentle trituration with a Pasteur pipette resulted in isolated cells mixed with cartilage nodules. This mixture was plated as an organ/cell culture in 1:1 (vol/vol) high-glucose Dulbecco's modified Eagle's medium (DMEM; JRH Biosciences, Lenexa, KS)/Ham's F-12 (GIBCO) supplemented with 2 mM L-glutamine (GIBCO), 10% fetal bovine serum (GIBCO), and antibiotics (penicillin at 100 units/ml, streptomycin at 100 μg/ml; GIBCO) on 100-mm dishes (Corning). The same medium was used for all the derived cultures, and cells were grown at 37°C under 5% CO_2.

Retroviral Infection. The ψ2-865 cell line producing the ecotropic helper-free retrovirus was provided by P. A. Sharp (Massachusetts Institute of Technology, Cambridge). This replication-defective retrovirus encodes the SV40 large T antigen and a protein conferring resistance to neomycin (Fig. 1). Twenty-four hours after plating, the organ/cell culture was infected for 2 hr with the retrovirus supernatant in the presence of Polybrene (Aldrich) at 8 μg/ml. After infection, fresh medium was added, and 48 hr later the cultures were subjected to selection for 1 month in the presence of G418 (Geneticin; GIBCO) at 200 μg/ml. The selective medium was changed every 3–4 days. After 1 month, several colonies of cells showing a polygonal morphology were isolated in 35-mm wells by using cloning rings, and three populations of cells were found to give positive immunofluorescence with an antibody against type II collagen. One population was able to produce nodules after reaching confluence, and this was progressively enriched for chondrocyte-like cells after a series of trypsinizations on the same dish to remove fibroblast-like cells. The cells from this population were then passed at high dilution (about 20 cells per 100-mm dish), and 24 resulting colonies were isolated. Five colonies were selected on the basis of morphological criteria. A second population was also subcloned at high dilution and one colony was selected. Cells from the six clones (five from the first population and one from the second population) stained with an anti-type II collagen antibody and a large T antigen antibody by immunofluorescence. One clone (clone MC615) was expanded further, and is the cell line described in this paper.

Cell Growth. Cells from passage 17 were plated in 35-mm wells (20,000 cells per well). At the indicated days, cells were trypsinized and counted in triplicate with a hemocytometer.

Analysis of RNA and Genomic DNA. Total RNA extracted from cultures with guanidine isothiocyanate (19), electrophoresed in agarose gels, and transferred to nylon N membranes (Amersham) was hybridized with probes labeled with [^{32}P]dCTP by random priming (20). The following DNA

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Abbreviation: SV40, simian virus 40.

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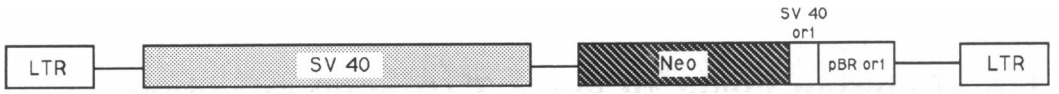

FIG. 1. The recombinant pZipNeoSV40, containing the wild-type SV40 early regions (nucleotides 5235–2666) inserted at the *Bam*HI site of the pZipNeoSV(X)I shuttle vector (18). LTR, long terminal repeat; ori, origin of replication.

probes were used: (*i*) a clone (pEVEHO.4) containing a 321-bp *Eco*RI–*Hin*dIII fragment of the mouse α1(I) collagen gene, corresponding mainly to the 3′ untranslated region of the α1(I) mRNA (21), a gift from E. Vuorio (M. D. Anderson Cancer Center, Houston, TX); (*ii*) a clone containing a 405-bp *Eco*RI–*Hin*d III fragment of the mouse α1(II) collagen gene, corresponding mainly to the 3′ untranslated sequence of the mRNA (21), also a gift from E. Vuorio; (*iii*) a clone (pYMm128) containing a 1-kb *Eco*RI fragment of mouse α1(IX) collagen cDNA encoding the NC3 and a small portion of the COL3 domains, provided by Y. Muragaki (Wakayama Medical College, Wakayama, Japan); (*iv*) a clone (pVLm8) containing a 500-bp *Xba* I–*Xho* I fragment corresponding to part of the 3′ untranslated region of the mouse α2(XI) collagen gene, provided by K. Cheah (University of Hong Kong); (*v*) a clone (p1355) containing a 710-bp *Pst* I cDNA fragment coding for the carboxyl end of the rat aggrecan core protein (22), provided by Y. Yamada (National Institute of Dental Research, Bethesda, MD); (*vi*) a clone (p1356) containing a 820-bp *Eco*RI cDNA fragment encoding the carboxyl ⅔ of the rat link protein (23, 24), also a gift from Y. Yamada; and (*vii*) a clone (I-19) containing a 4.8-kb *Sal* I–*Eco*RI gene fragment corresponding to the mouse 28S RNA coding sequence (25), provided by N. Arnheim (University of Southern California, Los Angeles). Northern hybridizations were performed in the presence of 5× SSPE, 5× Denhardt's solution, 50% (vol/vol) formamide, salmon sperm DNA at 180 μg/ml, and 0.1% SDS at 42°C overnight (1× SSPE = 150 mM NaCl/10 mM sodium phosphate, pH 7.4/1 mM EDTA; 1× Denhardt's solution = 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone). The washing conditions were 2× SSC at room temperature and 0.5× SSC, 0.2× SSC, 0.1× SSC at 60°C, in the presence of 0.1% SDS each time (1× SSC = 150 mM NaCl/15 mM sodium citrate, pH 7). Genomic DNA was isolated by using a simplified standard procedure (26). The pZipSV7761 plasmid (derived from a pZipNeoSV40 plasmid as shown in Fig. 1), provided by P. Jat (Ludwig Institute for Cancer Research, London), was used to generate two DNA probes to analyze viral DNA insertions into genomic DNA: (*i*) a *Bam*HI fragment specific for the SV40 early region; and (*ii*) a *Xho* I fragment specific for the sequence conferring resistance to neomycin. The probes were labeled with [^{32}P]dCTP by random priming. Southern hybridizations on Hybond N+ membranes (Amersham) were performed in the presence of 6× SSC/50 mM sodium phosphate, pH 6.8/50% formamide/5% dextran sulfate/salmon sperm DNA at 25 μg/ml/1 mM EDTA, pH 8/0.5% SDS/1× Denhardt's solution at 42°C overnight. The washing conditions were 2× SSC at room temperature and 0.1× SSC at 55°C, in the presence of 0.1% SDS each time.

Western Blotting. Cultures were grown in the presence of ascorbic acid (Sigma) at 50 μg/ml and β-aminopropionitrile fumarate (United States Biochemical, Cleveland) at 50 μg/ml for 48 hr before extraction of the medium and of the cell layer. The medium was dialyzed against 0.5 M acetic acid, digested with pepsin (2650 units/mg; Sigma) at 0.5 mg/ml overnight at 4°C, and lyophilized, and a portion of the sample was digested with bacterial collagenase (50 units/ml; Advance Biofactures, Lynnbrook, NY) for 2 hr at 37°C before Western blotting with the anti-type II collagen antibody. The medium was dialyzed against 0.5 M Tris·HCl, pH 7.2/0.05 M $CaCl_2$, and a portion of the sample was digested with bacterial collagenase before the reaction with the anti-type IX collagen antibody. The media and cell lysates were electrophoresed in SDS/polyacrylamide gels using standard procedures (27). The protein bands were electrotransferred to nitrocellulose (Schleicher & Schuell) and allowed to react with (*i*) a monoclonal antibody (2B1) against type II collagen (28), provided by R. Mayne (University of Alabama at Birmingham); (*ii*) a monoclonal antibody (23-5D1) specific for the α1(IX) chain (29), a gift of K. Iwata (Fuji Chemical Industries, Takaoka, Japan); and (*iii*) a monoclonal antibody (9/30/8-A-4) against link protein (30), obtained from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City). Detection was with secondary antibodies coupled to alkaline phosphatase (Promega) and a substrate kit from Bio-Rad.

Immunofluorescence and Staining. Cells were grown on Lab-Tech chamber slides (Nunc) and were fixed with 2.5% paraformaldehyde (J. T. Baker) in phosphate-buffered saline (PBS; Sigma) for 30 min at room temperature, rinsed in PBS, and treated with hyaluronidase (800 units/ml, Sigma type I) for 30 min at 37°C, before one of the following steps: (*i*) addition of the 2B1 antibody specific for type II collagen; (*ii*) permeabilization with Triton X-100 (Sigma) and addition of a monoclonal antibody (pAb 419) with specificity for antigenic determinants in the amino-terminal domain of the SV40 large T antigen (31), a gift of J. A. DeCaprio (Dana–Farber Cancer Institute, Boston); or (*iii*) permeabilization with Triton X-100 and addition of a polyclonal antibody specific for type I collagen, a gift from L. Iruela-Arispe and H. Sage (University of Washington, Seattle). Cells were fixed with cold 95% ethanol overnight and dehydrated with cold 100% ethanol before treatment with hyaluronidase and addition of a polyclonal antibody recognizing the NC4 domain of α1(IX) collagen, provided by M. T. McCarthy (Harvard Medical School, Boston).

To detect the sulfated chains of aggrecan, cultures were rinsed in PBS, fixed in Kahle's fixative (32) for 30 min, and rinsed again in PBS. Sulfated glycosaminoglycans were detected by staining with Alcian blue at pH 1 overnight (33).

Tumorigenicity Assay. Five-week-old female BALB/c *nu/nu* mice (Charles River Breeding Laboratories) were injected with 6–7 × 10^6 cells subcutaneously in the scapular

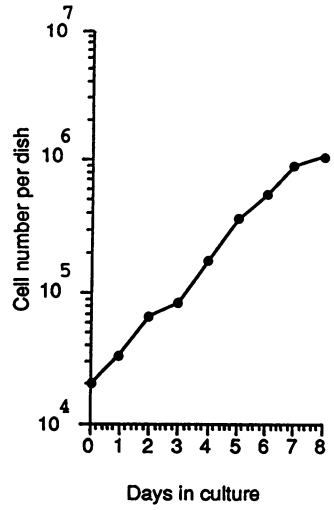

FIG. 2. Growth curve of MC615 plated at passage 17 in DMEM/Ham's F-12 supplemented with 10% fetal bovine serum.

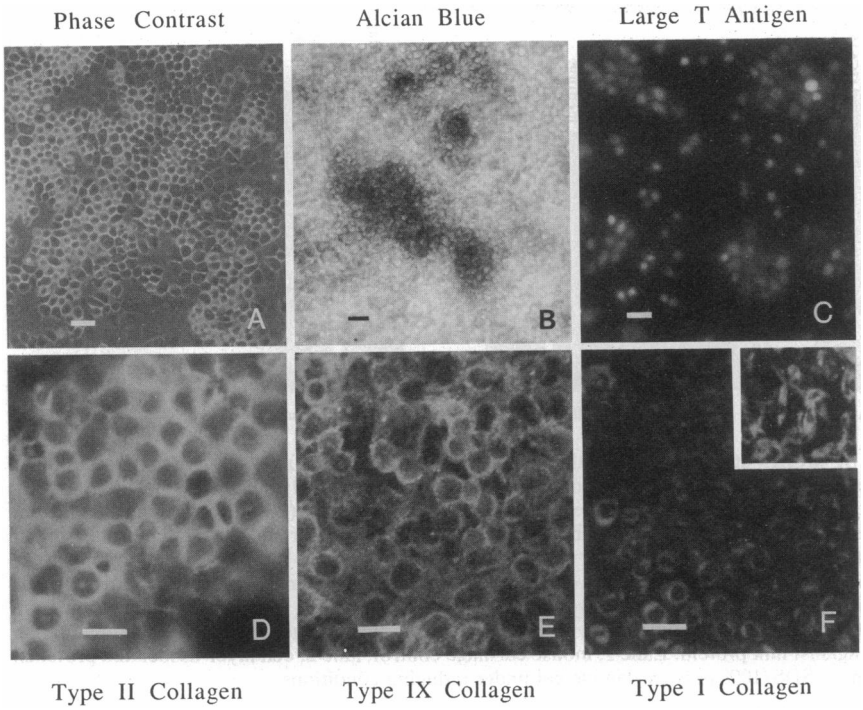

FIG. 3. Morphology and staining of MC615 plated on culture dishes. (*A*) Cells show a polygonal morphology, with a refractile matrix. (*B*) After reaching confluency, cells form nodules that stain with Alcian blue. (*C*) Staining of the nuclei with the pAb 419 antibody directed against the amino-terminal domain of SV40 large T antigen. (*D*) Staining of the matrix with an anti-type II collagen antibody (2B1). (*E*) Staining of the matrix with an anti-type IX collagen antibody. (*F*) Staining with an anti-type I collagen antibody, after permeabilization of the cells with Triton X-100. Intracellular vesicles show a weak fluorescence when compared with mouse skin fibroblasts stained with the same antibody (*Inset*). (Bars = 20 μm.)

region. The mice were examined weekly for 2 months for the presence of tumors and then autopsied.

RESULTS

Growth Properties and Morphology. The MC615 cells grew on tissue culture dishes with a doubling time of about 28 hr (Fig. 2). In monolayer, the cells showed a polygonal shape (Fig. 3*A*). After reaching confluency the cells formed nodules which stained with Alcian blue at pH 1, indicating the presence of sulfated aggrecan (Fig. 3*B*). No signs of cell hypertrophy were observed in the cultures, even after nodule formation. The MC615 cells have been passaged some 20 times (during at least 7 months), without any sign of senescence, and were able to grow in suspension in bacterial dishes, forming multicellular aggregates (data not shown). SV40 large T antigen was visualized by indirect immunofluorescence in all MC615 cells (Fig. 3*C*).

Expression of the Chondrocyte Phenotype. The matrix produced by MC615 cells appeared highly refractile by phase-contrast microscopy once the cells reached confluency and showed positive staining with an anti-type II collagen antibody (Fig. 3*D*). Positive staining was also seen with an antibody against the NC4 domain of type IX collagen (Fig. 3*E*) (34). A low level of type I collagen was detected in some chondrocytes by immunofluorescence (Fig. 3*F*). Northern blot analysis confirmed these results: pro-α1(II), α1(IX), and pro-α1(I) transcripts were detected (Fig. 4). Moreover, α2(XI) mRNA (35), as well as aggrecan and link protein mRNAs, was detected (Fig. 4). No signal was detected after hybridization with a mouse α1(X) cDNA probe (36), even after 10 days of exposure (data not shown). Western blotting

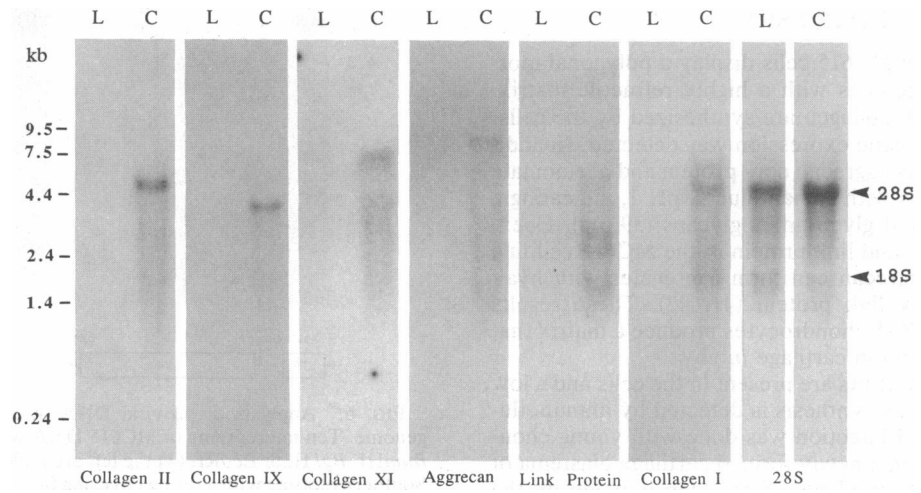

FIG. 4. Northern blot hybridization of total RNAs isolated from newborn mouse liver (L) or from MC615 chondrocytes (C). About 20 μg of each RNA was fractionated by electrophoresis in a 1% agarose gel in the presence of Mops buffer. The membrane was hybridized with probes as indicated below each panel. After each hybridization, the membrane was stripped of the probe and rehybridized. Exposure times at −70°C were 13 hr for α1(II), 20 hr for α1(IX), 4 days for α2(XI), 13 hr for aggrecan, 3 days for link protein, and 24 hr for α1(I). Exposure time of 25 min at room temperature for the 28S RNA probe reveals a stronger signal in liver (L) than in MC615 (C), suggesting that more RNA was loaded in MC615 chondrocytes. To examine the tissue specificity of the signals, autoradiographs have been exposed for longer times than those indicated (up to 10 days, data not shown), but none of the transcripts studied here have been detected in the liver. The positions of RNA size markers are shown on the left; the positions of 28S and 18S ribosomal RNAs are shown on the right.

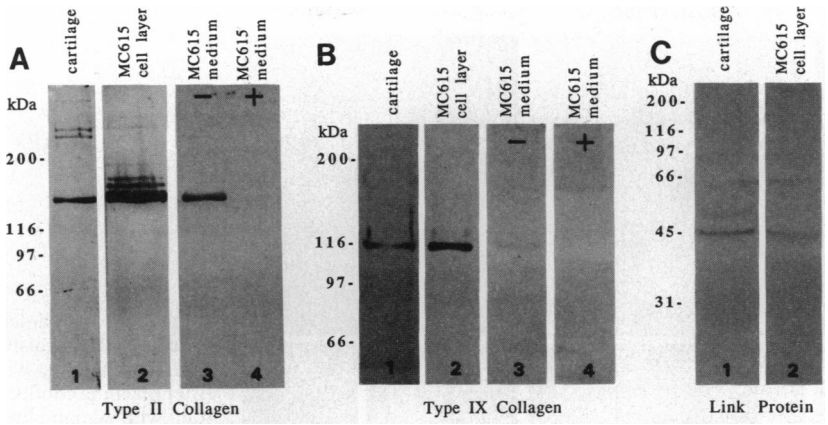

FIG. 5. Expression of cartilage matrix proteins by MC615 at passage 17, in the cell layer and in the medium. The positions of globular molecular size markers are shown on the left side of each panel. Since collagenous polypeptides migrate more slowly in SDS/PAGE than globular proteins, the globular markers do not indicate their true molecular sizes. (*A*) Western blotting using an antibody (2B1) against type II collagen. Lane 1, mouse cartilage control; lane 2, cell layer-associated proteins (the upper bands probably represent intermediates in the processihg of type II procollagen); lane 3, medium after pepsinization; and lane 4, medium after pepsinization and collagenase treatment. The proteins were fractionated by electrophoresis in an SDS/6% polyacrylamide gel under reducing conditions. (*B*) Western blotting using an antibody (23-5D1) directed against type IX collagen. Lane 1, mouse cartilage control; lane 2, cell layer-associated proteins; lane 3, medium; and lane 4, medium after collagenase treatment. The proteins were fractionated by electrophoresis in an SDS/6% polyacrylamide gel under reducing conditions. (*C*) Western blotting using an antibody (9/30/8-A-4) against link protein. Lane 1, mouse cartilage control; lane 2, cell layer-associated proteins. The proteins were fractionated by electrophoresis in an SDS/10% polyacrylamide gel under reducing conditions.

showed high levels of type II collagen (Fig. 5*A*) and of the long form of type IX collagen (Fig. 5*B*) associated with the cell layer, and also expression of the link protein (Fig. 5*C*).

Southern blotting was used to determine whether the cell line was clonal (Fig. 6). The SV40 fragment released by *Bam*HI digestion is too small to encode the small t antigen (37, 38), showing that the retrovirus used in this study transduces only the large T antigen. *Xba* I cuts in the viral long terminal repeat sequences, releasing a full-length proviral sequence. *Eco*RI and *Bgl* II cut once in the provirus, yielding only a single fragment containing SV40 sequences and flanking cellular DNA (Fig. 6*A*). The same membrane hybridized with a neomycin resistance probe confirms that the MC615 cells carry only a single viral copy at the insertion site (Fig. 6*B*).

Tumorigenicity. No tumors were found after 2 months in any of the five mice injected with the MC615 chondrocytes.

DISCUSSION

In monolayer culture MC615 cells display a polygonal morphology and form nodules with a highly refractile matrix. Both types II and IX collagen are synthesized by the cells, and α2(XI) collagen gene expression was detected. In addition, the cells express aggrecan core protein and accumulate a matrix which stains with Alcian blue at pH 1, indicating a matrix rich in sulfated glycosaminoglycans (39, 40). Coexpression of aggrecan and link protein in the MC615 cell line suggests that the aggrecan can form aggregates with hyaluronan stabilized by link protein (41, 42). These results indicate that the MC615 chondrocytes produce a matrix that is similar to that found in cartilage *in vivo*.

α1(I) collagen transcripts are present in the cells and a low level of type I collagen synthesis is detected by immunofluorescence. Retroviral infection was done with young chondrocytes isolated from a newly formed cartilage blastema of the limb bud (day 14) and type I collagen is made by the blastema before deposition of the cartilage matrix (43). Also, articular chondrocytes express type I collagen at early stages of development (44, 45), and it is possible that the clonal MC615 cell line is derived from a particular precursor cell committed to some type I collagen expression. It is also possible, however, that type I collagen expression is a culture artefact. Rat chondrocytes immortalized by a retrovirus carrying the *myc* and *raf* oncogenes express α2(I) collagen in monolayer but not in suspension culture (15), and primary chondrocytes start to express type I collagen when maintained in monolayer culture (9–11).

The MC615 cell line was not tumorigenic in five injected mice and the MC615 cells in culture presented evidence of

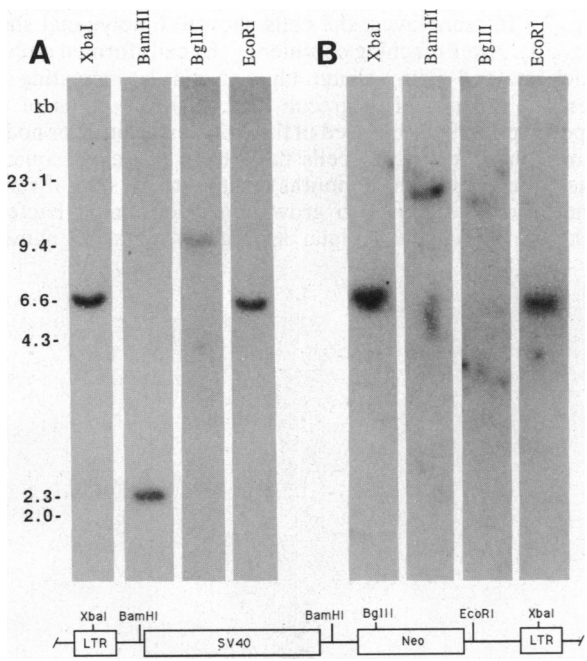

FIG. 6. Analysis of proviral DNA integrated into the MC615 genome. Ten micrograms of MC615 DNA was digested with *Xba* I, *Bam*HI, *Bgl* II, or *Eco*RI. On the left are molecular size markers. (*A*) Southern blotting with a probe covering the SV40 insert. (*B*) Analysis of the same filter with a probe covering the neomycin-resistance (Neo) sequence. At the bottom is a schematic representation of the integrated proviral DNA. Since *Bgl* II cuts within the Neo sequence, hybridization of the *Bgl* II digest with the Neo probe gives two bands: one of the same size as the band visualized after hybridization with the SV40 probe (*A*; this band is not seen on the autoradiogram *B* and is visible only after longer exposure; data not shown), and a second band of different size, visible here below 23.1 kb (*B*).

some contact inhibition before nodule formation. This is in agreement with studies showing that the SV40 large T antigen gene is not an acute transforming gene (17, 38). The mechanism by which SV40 large T antigen has immortalized the MC615 cells is not known, but at least two known regulatory proteins that control the cell cycle, the retinoblastoma (Rb) and p53 proteins, are potential targets for large T activity. The large T oncoprotein is able to bind to the unphosphorylated form of Rb, abolishing the growth-suppressive function of Rb (46–49), and large T antigen can also complex with p53 (50, 51), a protein whose expression is not compatible with continued growth (52). Thus, loss of p53 expression was observed in several immortalized chicken cell lines (53).

Changes in type II collagen expression in rabbit chondrocytes immortalized by SV40 early functions (54) or in rat chondrocytes immortalized by *myc* have been reported (15). These observations and our data suggest that SV40 large T (or *myc*) expression can maintain chondrocytes in a proliferative state while the cartilage phenotype is retained at various levels.

Type X collagen expression and signs of hypertrophy were not found in cells stabilized by *myc* (12–15). In our experiments, the MC615 cells have been grown in the presence of 10% fetal bovine serum, known to induce hypertrophy and type X collagen synthesis in chicken chondrocytes (55), but no signs of hypertrophy or type X collagen expression was detected in the MC615 cultures. This suggests that expression of large T or *myc* oncogenes, while maintaining chondrocytes in their proliferative state, prevents them from entering the hypertrophic phase.

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1. Comper, W. D. (1991) in *Cartilage: Molecular Aspects*, eds. Hall, B. & Newman, S. (CRC, Boca Raton, FL), pp. 59–96.
2. Heinegard, D. & Oldberg, A. (1989) *FASEB J.* **3**, 2042–2051.
3. Upholt, W. B. & Olsen, B. R. (1991) in *Cartilage: Molecular Aspects*, eds. Hall, B. & Newman, S. (CRC, Boca Raton, FL), pp. 43–79.
4. Wight, T. N., Heinegard, D. K. & Hascall, V. C. (1991) in *Cell Biology of Extracellular Matrix*, ed. Hay, E. E. (Plenum, New York), pp. 45–78.
5. Mendler, M., Eich-Bender, S. G., Vaughan, L., Winterhalter, K. H. & Bruckner, P. (1989) *J. Cell Biol.* **108**, 191–197.
6. Vaughan, L., Mendler, M., Huber, S., Bruckner, P., Winterhalter, K. H., Irwin, M. I. & Mayne, R. (1988) *J. Cell Biol.* **106**, 991–997.
7. Jacenko, O., Olsen, B. R. & LuValle, P. (1991) *Crit. Rev. Eukaryotic Gene Expression* **1**, 327–353.
8. Gerstenfeld, L. C. & Landis, W. J. (1991) *J. Cell Biol.* **112**, 501–513.
9. Mayne, R., Vail, M., Mayne, P. M. & Miller, E. J. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 1674–1678.
10. von der Mark, K., Gauss, V., von der Mark, H. & Müller, P. (1977) *Nature (London)* **267**, 531–532.
11. Benya, P. D., Padilla, S. R. & Nimni, M. E. (1978) *Cell* **15**, 1313–1321.
12. Alema, S., Tato, F. & Boettiger, D. (1985) *Mol. Cell. Biol.* **5**, 538–544.
13. Gionti, E., Pontarelli, G. & Cancedda, R. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 2756–2760.
14. Quarto, R., Dozin, B., Tacchetti, C., Robino, G., Zenke, M., Campanile, G. & Cancedda, R. (1992) *Dev. Biol.* **149**, 168–176.
15. Horton, W. E., Cleveland, J., Rapp, U., Nemuth, G., Bolander, M., Doege, K., Yamada, Y. & Hassell, J. R. (1988) *Exp. Cell Res.* **178**, 457–468.
16. Takigawa, M., Pan, H.-O., Kinoshita, A., Tajima, K. & Takano, Y. (1991) *Int. J. Cancer* **48**, 717–725.
17. Jat, P. S. & Sharp, P. A. (1986) *J. Virol.* **59**, 746–750.
18. Cepko, C. L., Roberts, B. E. & Mulligan, C. (1984) *Cell* **37**, 1053–1062.
19. Chirgwin, J. J., Przybyla, A. E., MacDonald, R. J. & Rutter, W. J. (1979) *Biochemistry* **18**, 5294–5299.
20. Feinberg, A. P. & Vogelstein, B. (1984) *Anal. Biochem.* **137**, 266–267.
21. Metsäranta, M., Toman, D., DeCrombrugghe, B. & Vuorio, E. (1991) *Biochim. Biophys. Acta* **1089**, 241–243.
22. Doege, K., Fernandez, P., Hassell, J. R., Sasaki, M. & Yamada, Y. (1986) *J. Biol. Chem.* **261**, 8108–8111.
23. Doege, K., Hassell, J. R., Caterson, B. & Yamada, Y. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 3761–3765.
24. Rhodes, C., Doege, K., Sasaki, M. & Yamada, Y. (1988) *J. Biol. Chem.* **263**, 6063–6067.
25. Arnheim, N. (1979) *Gene* **7**, 83–96.
26. Laird, P. W., Zijderveld, A., Linders, K., Rudnicki, M. A., Jaenisch, R. & Berns, A. (1991) *Nucleic Acids Res.* **19**, 4293–4294.
27. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
28. Fitch, J. M., Mentzer, A., Mayne, R. & Linsenmayer, T. F. (1988) *Dev. Biol.* **128**, 396–405.
29. Warman, M., Kimura, T., Muragaki, Y., Castagnola, P., Tamei, H., Iwata, K. & Olsen, B. R. (1992) *Matrix*, in press.
30. Caterson, B., Baker, J. R., Christner, J. E., Lee, Y. & Lentz, M. (1985) *J. Biol. Chem.* **260**, 11348–11356.
31. Harlow, E., Crawford, L. V., Pim, D. C. & Williamson, N. M. (1981) *J. Virol.* **39**, 861–869.
32. Ahrens, P., Solursh, M. & Reiter, R. (1977) *Dev. Biol.* **60**, 69–82.
33. Lev, R. & Spicer, S. (1964) *J. Histochem. Cytochem.* **12**, 309.
34. Vasios, G., Nishimura, I., Konomi, H., van der Rest, M., Ninomiya, Y. & Olsen, B. R. (1988) *J. Biol. Chem.* **263**, 2324–2329.
35. Kimura, T., Cheah, K. S. E., Chan, S. D. H., Lui, V. C. H., Mattei, M.-G., van der Rest, M., Ono, K., Solomon, E., Ninomiya, Y. & Olsen, B. R. (1989) *J. Biol. Chem.* **264**, 13910–13916.
36. Apte, S. S., Seldin, M. F., Hayashi, M. & Olsen, B. R. (1992) *Eur. J. Biochem.* **206**, 217–224.
37. Berk, A. J. & Sharp, P. (1978) *Proc. Natl. Acad. Sci. USA* **3**, 1274–1278.
38. Jat, P. S., Cepko, C. L., Mulligan, R. C. & Sharp, P. A. (1986) *Mol. Cell. Biol.* **6**, 1204–1217.
39. Capasso, O., Gionti, E., Pontarelli, G., Ambesi-Impiombato, F. S., Nitsch, L., Tajana, G. & Cancedda, R. (1982) *Exp. Cell Res.* **142**, 197–206.
40. Hassel, J. R. & Horigan, E. A. (1982) *Teratog. Carcinog. Mutagen.* **2**, 325–331.
41. Heinegard, D. & Hascall, V. C. (1974) *J. Biol. Chem.* **249**, 4250–4256.
42. Falts, L. L., Caputo, C. B., Kimura, J. H., Schrode, J. & Hascall, V. C. (1979) *J. Biol. Chem.* **254**, 1381–1387.
43. von der Mark, K. (1980) *Curr. Top. Dev. Biol.* **14**, 199–225.
44. Sandberg, M., Mäkelä, J. K., Multimäki, P., Vuorio, T. & Vuorio, E. (1989) *Matrix* **9**, 82–91.
45. Treilleux, I., Mallein-Gerin, F., Le Guellec, D. & Herbage, D. (1992) *Matrix* **12**, 221–232.
46. DeCaprio, J. A., Ludlow, J. W., Figge, J., Shew, J. Y., Huang, C. M., Lee, W. H., Marsilio, E., Paucha, E. & Livingston, D. M. (1988) *Cell* **54**, 275–283.
47. Ludlow, J. W., DeCaprio, J. A., Huang, C. M., Lee, W. H., Paucha, E. & Livingston, D. M. (1989) *Cell* **56**, 57–65.
48. Kaelin, W. G., Erwen, M. E. & Livingston, D. M. (1990) *Mol. Cell. Biol.* **10**, 3761–3769.
49. Ludlow, J. W., Shon, J., Pipas, J. M., Livingston, D. M. & Caprio, J. A. (1990) *Cell* **60**, 387–396.
50. Linzer, D. I. H. & Levine, A. J. (1979) *Cell* **17**, 43–52.
51. Lane, D. P. & Crawford, L. V. (1979) *Nature (London)* **278**, 261–263.
52. Johnson, P., Gray, D., Mowat, M. & Benchimol, S. (1991) *Mol. Cell. Biol.* **11**, 1–11.
53. Ulrich, E., Boehmelt, G., Bird, A. & Beug, H. (1992) *Genes Dev.* **6**, 876–887.
54. Thenet, S., Benya, P. D., Demignot, S., Feunteun, J. & Adolphe, M. (1992) *J. Cell. Physiol.* **150**, 158–167.
55. Bruckner, P., Horler, I., Mendler, M., Houze, Y., Winterhalter, K. H., Eich-Bender, S. G. & Spycher, M. A. (1989) *J. Cell Biol.* **109**, 2537–2545.