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Avian myelocytomatosis virus immortalizes differentiated quail chondrocytes

(v-*myc* gene/cartilage extracellular matrix/type X collagen)

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ABSTRACT **Quail embryo chondrocytes in culture display two morphological phenotypes: polygonal epithelial-like and floating cells. Both cell populations synthesize cartilage extracellular matrix proteins (type II collagen and specific proteoglycans), whereas type X collagen, which appears to be a marker of later stages of chondrocyte differentiation, is expressed only by the epithelial-like cells. Avian myelocytomatosis virus strain MC29 does not induce morphological transformation in quail embryo chondrocytes but stimulates these cells to proliferate with a progressively reduced doubling time. MC29-infected chondrocytes can be established in culture as a continuous cell line, whereas control (uninfected) cultures only survive a few months. Rapidly dividing MC29-infected chondrocytes still express type II collagen and cartilage proteoglycans but do not synthesize type X collagen.**

The replication-defective acute leukemia virus, avian myelocytomatosis virus strain MC29, induces a variety of tumors in birds and transforms fibroblasts and macrophages in culture (1); responsible for these effects is the only known viral gene product (2), the DNA-binding protein $p110^{gag\text{-}myc}$ (3). $p110^{gag\text{-}myc}$ is a fusion protein containing an NH_2-terminal portion, specified by the *gag* structural gene, linked to the product of the v-*myc* gene (4, 5). Alterations in the expression and in the structure of c-*myc*, the cellular counterpart of v-*myc* (6, 7), have been observed in malignant neoplasms in chickens (8, 9), mice, and humans (10–17). The *myc* gene can cooperate with the Ej-Ha-*ras* oncogene to convert primary fibroblasts into tumorigenic cells, supplying a function essentially similar to the DNA virus transforming genes large T of polyoma virus (18) and E *1A* of adenovirus (19). These functional homologies, and some sequence homology between v-*myc* and E *1A* (20), suggest that *myc* behaves as an "immortalizing gene," while a second or possibly other oncogenes contribute other aspects of malignant transformation (21–23). Furthermore, increased expression of c-*myc* has been shown in the early stage of growth stimulation in different cell types (24, 25), suggesting for c-*myc* a function related to the progression of cells through the growth cycle.

Cultured avian embryo chondrocytes represent a suitable system for study of the modulation of the differentiated phenotype in proliferating cells. These cells, in fact, divide for many generations while expressing cartilage extracellular matrix proteins. We describe the characterization of quail embryo chondrocyte culture and report the effect of v-*myc* on cell proliferation and on the expression of the differentiated program in this system.

MATERIALS AND METHODS

Cell Culture and Viral Infection. Chondrocytes were obtained from 13-day Japanese quail embryo tibiae, plated at low density (10^3 cells per cm^2) in Coon's modified F-12 medium supplemented with 10% fetal calf serum (GIBCO), and kept in culture essentially as described for chick embryo chondrocytes (26). Quail embryo fibroblasts were obtained from 8-day Japanese quail embryos and grown in Dulbecco's modified Eagle's medium containing 10% tryptose phosphate broth (Difco), 4% calf serum (GIBCO), and 1% chicken serum (GIBCO).

Epithelial-like chondrocytes grown in the absence of ascorbic acid were detached from the dish with trypsin and infected in suspension in a small volume at a multiplicity of infection ≈20 with Rous-associated virus 1 (RAV-1) or MC29 (RAV-1) pseudotype. Infected and mock-infected chondrocytes were replated (5×10^4 cells per 35-mm dish) and fresh medium was added.

[^{35}S]Methionine Labeling, Immunoprecipitation, and Polyacrylamide Gel Electrophoresis. Labeling, immunoprecipitation, and PAGE were done as described (26). Rabbit antibodies against chicken type II collagen were generously provided by B. Vertel (Syracuse University) (27).

Immunofluorescence. Normal and MC29-infected chondrocytes or fibroblasts were plated on coverslips, and immunofluorescence staining was performed as described (28). Ascorbic acid was added to a final concentration of 50 μg/ml 24 hr before staining. Rabbit antibodies against the core protein of chick cartilage-specific proteoglycans were a gift from B. Vertel (27).

RNA Extraction and Dot Blot Hybridization. Total cellular RNA was extracted by the guanidine·HCl procedure (29). To assess the integrity of RNA, 5 μg of total RNA from each sample was denatured, fractionated on formaldehyde/agarose gels, and stained with ethidium bromide. Samples containing 5, 2, and 1 μg of total cell RNA each with, respectively, 0, 3, and 4 μg of carrier RNA (total yeast RNA, BDH) were dotted in 4 μl of 10× NaCl/Cit onto nitrocellulose paper previously equilibrated with 20× NaCl/Cit (1× NaCl/Cit is 0.15 M NaCl/15 mM sodium citrate, pH 7 (30). A molecular probe from the chicken c-*myc* locus subcloned in pBR322 (31) (kindly provided by W. Hayward, Memorial Sloan-Kettering Cancer Center, New York) was used for hybridization. The probe insert (1.8 kilobase pairs) spans from the *Sal* I site in the second exon to the end of the third exon. High specific activity nick-translated probe (1×10^8 cpm/μg) was prepared as described by Maniatis *et al.* (32) by using [α-^{32}P]dCTP and [α-^{32}P]dATP (Amersham). Prehybridization was carried out at 37°C in 50% (vol/vol) formamide/5× NaCl/Cit/50 mM sodium phosphate, pH 6.8/0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% bovine serum albumin/0.2% $NaDodSO_4$ containing yeast RNA at 250 μg/ml. The prehybridization mixture was replaced with

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Abbreviations: RAV, Rous-associated virus; QEC(MC29), quail embryo chondrocytes infected with avian myelocytomatosis virus strain MC29.

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fresh solution of the same composition but containing 12.5% (wt/vol) dextran sulfate and 5×10^6 cpm of denatured probe. Hybridization was for 16 hr at 37°C. The filters were washed three times for 5 min at room temperature in 2× NaCl/Cit/0.1% $NaDodSO_4$ and then incubated twice in 0.1× NaCl/Cit/0.1% $NaDodSO_4$ at 37°C for 15 min. Washed filters were dried and exposed to Kodak XAR-5 films with intensifying screens.

RESULTS

Culture of Quail Embryo Chondrocytes and Expression of Differentiated Functions. Chondrocytes obtained from 13-day Japanese quail embryo tibiae and seeded at low density (10^3 cells per cm^2) give rise, with few exceptions, to primary cultures not contaminated by fibroblasts. Quail chondrocytes present two morphological phenotypes, epithelial-like cells (Fig. 1) and floating cells which, unlike chick chondrocytes (26), remain as two distinct cell populations; the interconversion between the two cell compartments is minimal. Quail chondrocytes grow in culture as differentiated cells until they stop dividing after a crisis period during which they maintain their specific morphological and biochemical characteristics. The pattern of intracellular and secreted proteins from both floaters and epithelial-like chondrocytes (Fig. 2, lanes 2, 3, 5, and 6) reveals the presence of the cartilage-specific procollagen chain proα1(II), whereas the expression of type X collagen (26, 33–35) is associated only with epithelial-like cells (lanes 3 and 6). Synthesis of type II collagen and cartilage-specific proteoglycans in quail chondrocytes is revealed also by indirect immunofluorescence staining (not shown).

MC29-Infected Quail Embryo Chondrocytes: Cell Growth and Expression of Differentiated Functions. Epithelial-like cells were infected with cloned stocks of a RAV-1 pseudotype of avian myelocytomatosis virus, MC29 (RAV-1). Viral infection does not induce morphological transformation but strongly promotes cell proliferation. Compared to the mock-infected and helper virus-infected cultures, MC29 infected chondrocytes—QEC(MC29)—release into the medium a great number of floater cells (Fig. 1, *Lower Left*). QEC(MC29) express proα1(II) collagen but do not synthesize type X collagen (Fig. 2, lanes 1 and 4).

MC29-infected chondrocytes were developed into long-term cultures (Fig. 1, *Lower Right*). So far they have been kept in culture for 16 months (about 70 passages). This cell line retains the ability to express type II collagen (Fig. 3*A*). The identification of type II collagen was based on its electrophoretic mobility and its immunoprecipitation by specific antibodies. In addition QEC(MC29) were positively stained by indirect immunofluorescence with antibodies against type II collagen (Fig. 4*A*). We do not have a good explanation for the differences in the relative contents of proα1(II), pCα-1(II), pNα1(II), and α1(II) chains in media of uninfected and infected chondrocytes (Fig. 3*A*). We observed that, in most cases, cultures of cells at higher densities release into the medium greater amounts of unprocessed chains than cultures of lower cell density. Since virus-infected chondrocytes grow to a higher cell density than uninfected chondrocytes this could be a possible explanation for the differences. The unidentified collagenase-sensitive protein (arrowhead in Figs. 2 and 3*A*) does not comigrate with any type I and type II collagen chain and is present in both normal and MC29-infected quail chondrocytes.

Long-term cultured QEC(MC29) do not synthesize type X collagen (Fig. 3*A*). This collagen represents the major protein secreted by mock- and RAV-1-infected epithelial-like cells even when, 5 months after the primary culture, these cells underwent a crisis period resulting in reduced growth rate (Fig. 3*B*).

One year after infection, QEC(MC29) could still be stained with Alcian blue 8GX (C.I. 74240) at pH 2.5, which selectively stains proteoglycans both in cartilage tissue (36) and in cultured chondrocytes (26) (not shown). When the expression of cartilage-specific proteoglycans was re-examined 16

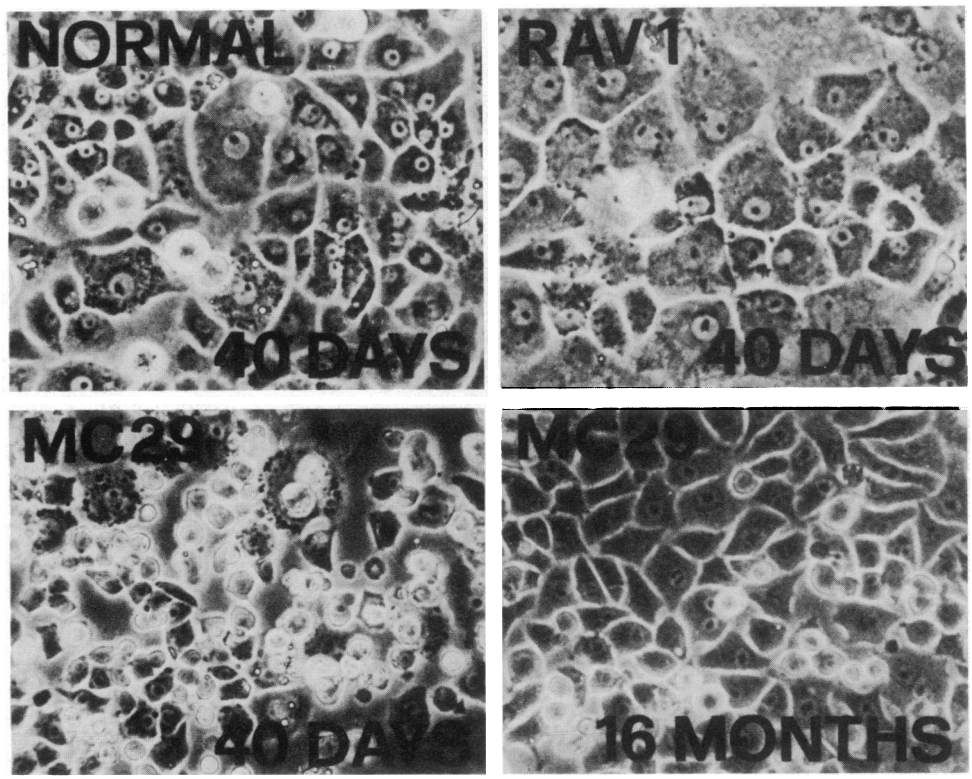

FIG. 1. Epithelial-like chondrocytes 40 days or 16 months after mock infection, infection by RAV 1 or infection by MC29 (RAV 1) pseudotype. (×240.)

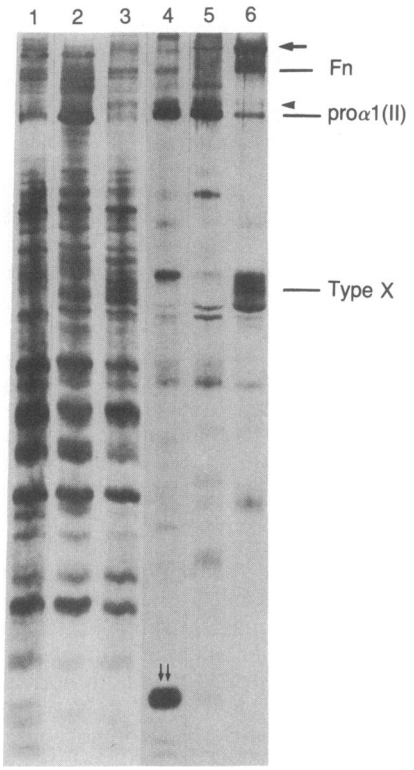

FIG. 2. Intracellular and secreted proteins from uninfected chondrocytes and from QEC(MC29) 40 days after infection. Cells were incubated with [^{35}S]methionine for 2 hr, and the proteins from cell lysates (lanes 1–3) and secreted into culture media (lanes 4–6) were analyzed by NaDodSO$_4$/9% PAGE and autoradiography. Lanes 1 and 4, QEC(MC29); 2 and 5, uninfected floaters; 3 and 6, uninfected epithelial-like cells. Fn, fibronectin; proα1(II), type II procollagen. Arrowhead points to an unidentified collagenase-sensitive protein (see text). The low M_r polypeptide (paired arrows) as well as the polypeptides migrating in the region of type X collagen in lane 4 are not collagenase-sensitive. Arrow points to an aggregated form of type X collagen.

months after infection, MC29-infected chondrocytes were still positively stained by indirect immunofluorescence with antibodies against the core protein of the cartilage-specific proteoglycans (Fig. 4*B*).

To verify whether the virus released by QEC(MC29) retains transforming ability, we collected cell media at different times after infection and found that 16 months after infection, QEC(MC29) still release infectious virus able to rapidly transform quail embryo fibroblasts. Moreover, QEC(MC29) express v-*myc* RNA at levels comparable to the levels observed in MC29-transformed quail fibroblasts (Fig. 5). As previously observed in MC29-transformed chick fibroblasts (37), MC29 does not induce plasminogen-activator synthesis in quail embryo chondrocytes (data not shown).

QEC(MC29) developed into long-term cultures without undergoing any crisis period of reduced growth rate. Rather, with increased time in culture, their doubling time progressively decreases to 19–20 hr (Fig. 6). QEC(MC29) are able to grow in medium supplemented with only 0.5% fetal calf serum. Control (uninfected) cultures proliferate only in medium supplemented with 10% fetal calf serum; in no case could we adapt them to grow in low-serum conditions.

DISCUSSION

In uninfected cultures from quail embryo tibiae, we detected two different morphological phenotypes, epithelial-like and floating chondrocytes. These two phenotypes display similar properties but also some distinctive features. Therefore, they seem to represent different cell compartments of the chondrocyte lineage. In contrast to what occurs with chick chondrocytes, in quail cultures the interconversion between the two morphological phenotypes is minimal, epithelial-like cells have a more limited lifespan than floating chondrocytes, and, more important, only epithelial-like chondrocytes express type X collagen. The location *in vivo* of chondrocytes competent for the synthesis of type X collagen (38, 39) indicates that this protein is synthesized by chondrocytes at late stages of differentiation. Taken together, these observations suggest that these two phenotypes could represent two distinct differentiation stages of cartilage cells.

v-*myc* immortalizes differentiated quail chondrocytes. Rapidly dividing MC29-infected chondrocytes still express type II collagen and cartilage-specific proteoglycans. We did not detect any type I collagen synthesis, whereas Rous sarcoma virus-induced transformation promotes the synthesis of this collagen in chick (40) and quail embryo chondrocytes (unpublished data). In both normal and MC29-infected quail chondrocytes, collagenase and limited pepsin digestions in-

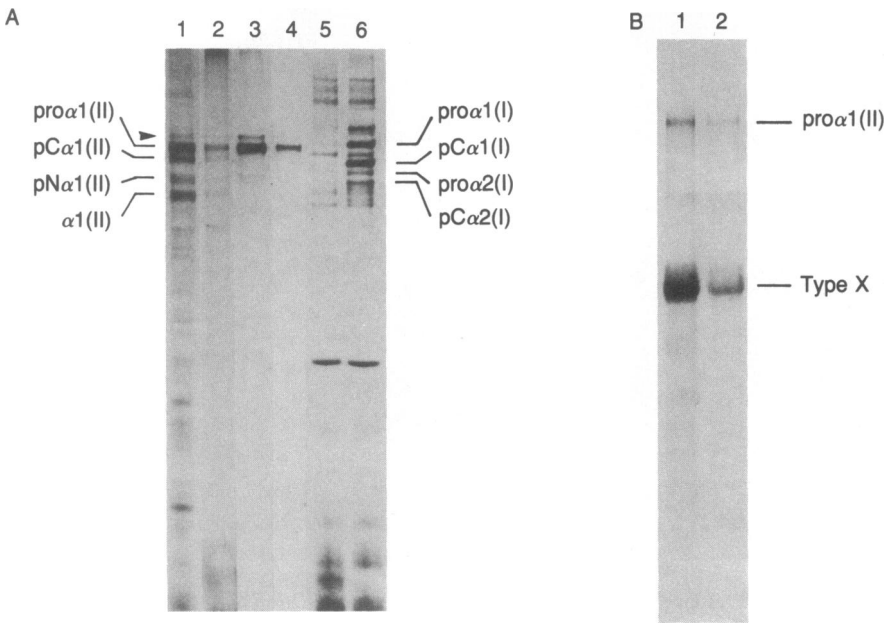

FIG. 3. Collagens synthesized by QEC(MC29) line. (*A*) Media from cultures incubated for 6 hr with [^{35}S]methionine were collected, and secreted proteins were analyzed by NaDodSO$_4$/7.5% PAGE. Lanes: 1, uninfected floating chondrocytes; 3, QEC(MC29) line; 2 and 4, immunoprecipitates (with rabbit antibodies to type II collagen) of proteins from uninfected floating chondrocytes (lane 2) and QEC(MC29) (lane 4); 5 and 6, proteins from normal quail embryo fibroblasts after and before collagenase digestion, respectively. Rabbit antibodies against chicken type II collagen precipitate poorly the intermediates in the processing of type II procollagen. (*B*) Proteins from mock- (lane 1) and RAV-1-infected (lane 2) epithelial-like cells. Cells were incubated for 2 hr with [^{35}S]methionine, and proteins secreted into the medium were trichloroacetic acid-precipitated and then analyzed by NaDodSO$_4$/9% PAGE.

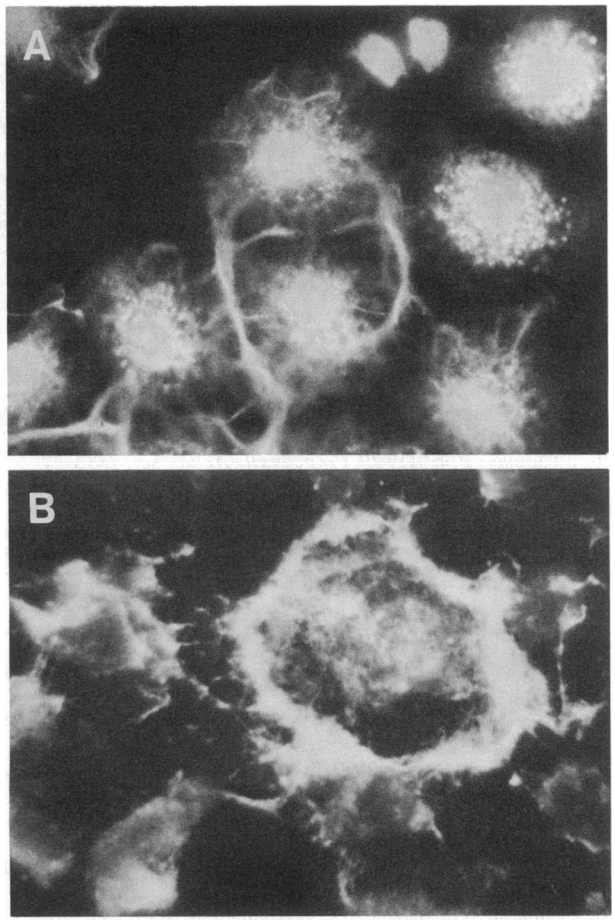

FIG. 4. Expression of type II collagen and cartilage-proteoglycan core protein in QEC(MC29). Cells were plated on coverslips and, after permeabilization with 0.1% Triton X-100, stained by indirect immunofluorescence with specific antibodies against type II collagen (*A*) or against cartilage-proteoglycan core protein (*B*). (×672.)

stead revealed other, unidentified minor collagens. MC29-infected epithelial-like chondrocytes do not synthesize type X collagen. The medium of virus-infected chondrocyte cultures contains a collagenase-insensitive low M_r polypeptide (Fig. 2) not found in the medium from uninfected epithelial-like chondrocytes. Previous work has shown that, when normal chondrocytes are metabolically labeled in the presence of ascorbic acid, type X collagen is converted into a low M_r

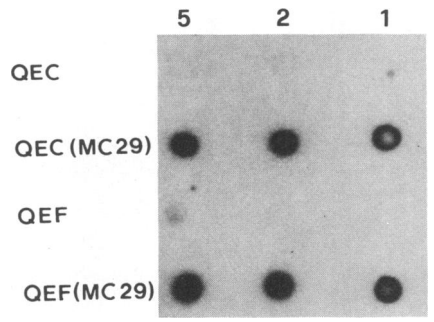

FIG. 5. Detection by dot blot hybridization, of *myc* specific sequences in RNA from normal and MC29-infected cells. Total RNA (5, 2, and 1 μg) was hybridized with a subclone of the chicken c-*myc* locus. QEF, quail embryo fibroblasts; QEF(MC29), MC29-transformed quail embryo fibroblasts; QEC, quail embryo chondrocytes; QEC(MC29), MC29-infected quail embryo chondrocytes.

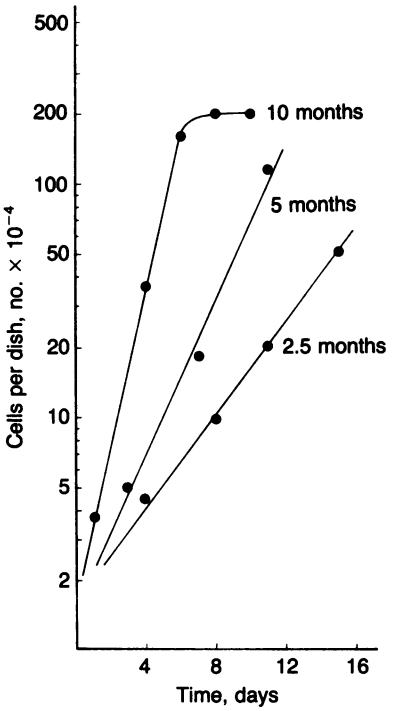

FIG. 6. Growth of quail chondrocytes at different times after MC29 infection. Cells were seeded at 2×10^4 cells per 30-mm dish 2.5, 5, and 10 months after infection. Cell number per dish at each indicated time after seeding was determined with a hemacytometer and represents the mean value of duplicate cultures.

fragment that is released into the medium (35); the M_r of this fragment is different from that released from QEC(MC29) and, furthermore, these cells were labeled in the absence of ascorbic acid.

The electrophoretic pattern of proteins from QEC(MC29) at different times after infection, even a few days after infection, are more similar to the pattern from uninfected "floaters" than to the one from epithelial-like cells. It is possible that some contaminating "floaters" could have been infected, rapidly expanded, and overgrown epithelial-like infected chondrocytes. However, we consider this unlikely because of the morphological appearance of cultures early after infection. Alternatively, one could speculate that v-*myc* product, while immortalizing epithelial-like cells, induces them to revert to earlier stages of chondrocyte differentiation by selective suppression of type X collagen expression. Whatever the interpretation, the immortalization of chondrocytes in a stage in which they do not express type X collagen further supports the idea that this collagen is a marker of later stages of differentiation.

Whereas control chondrocytes survive only few months, we observed a progressively reduced doubling time of MC29-infected cells. The increasing growth rate could be due to changes in the copy number of integrated viral DNA sequences and/or in their arrangement, as well as to changes in the expression or in the turnover of v-myc protein. Moreover, changes in the expression of cellular genes could have an additive effect on the regulation of growth rate by MC29.

With some exceptions (41–44), retrovirus-induced transformation prevents the expression of differentiation markers (40, 45–48). Hence, the expression of differentiated functions seems to be a primary target of viral oncogenes. We have shown previously that transformation by Rous sarcoma virus blocks the expression of chondrocyte-specific markers and promotes the synthesis of type I collagen and fibronectin (40), two glycoproteins made by the cartilage blastema before deposition of the specific extracellular matrix (49). Here

we report that, 16 months after infection, rapidly dividing MC29-infected chondrocytes still express differentiated functions but do not synthesize type X collagen. Whereas v-*src* seems to induce reversion of cultured chondrocytes to a predifferentiated state, v-*myc* immortalizes these cells without interfering with the expression of type II collagen and cartilage proteoglycans.

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