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Transformation of MMC-E Epithelial Cells by Acute 3611-MSV: Inhibition of Collagen Synthesis and Induction of Novel Polypeptides

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Mouse embryo epithelial cells MMC-E were transformed by novel fibrosarcoma-inducing murine sarcoma virus 3611-MSV. The cells were analyzed for the production and deposition of pericellular glycoproteins by immunofluorescence and by radioactive metabolic and cell surface labeling techniques followed by analysis in polyacrylamide gels and fluorography. The pericellular fibronectin matrix was lost, but unlike in virus-transformed fibroblastic cells, the production of fibronectin was not affected. The major differences detected were decrease in collagen production and initiation of synthesis of two major glycoproteins with M_r 58,000 and 60,000. Cell surface carbohydrate labeling indicated that after 3611-MSV transformation the cells expressed M_r 100,000 and 68,000 polypeptides. The present and previous results show that viral transformation of epithelial cells induces different transformed phenotypes that are associated with distinct alterations in pericellular glycoproteins.

Key words: epithelial cells, malignant transformation, 3611-MSV, procollagen, 58,000- and 60,000-dalton polypeptides

The loss of the pericellular matrix from cultured malignantly transformed cells has been commonly observed using the main matrix components fibronectin and collagen as markers [cf 1–3]. The disappearance of the pericellular matrix is quantitatively a major alteration of transformation, and several specific mechanisms that contribute to it have been described. These include decreased biosynthesis and processing of procollagen as well as altered production of specific proteinases [4–6]. The loss of the matrix has been most extensively studied in Rous sarcoma virus (RSV)-transformed cell systems including temperature-sensitive virus mutants [cf 7,8].

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Nontumorigenic mouse epithelial cells (MMC-E) have been isolated from an embryo of Mus musculus castaneous [9], and they contain cytokeratin type of intermediate filaments characteristic to epithelial cells only [10]. MMC-E cells can be transformed in vitro by mouse sarcoma virus (MSV), murine leukemia virus (C3H/MuLV), ethylnitrosourea, and polycyclic aromatic hydrocarbons [9,11–13]. In all cases the transformed cells were able to induce poorly differentiated neoplasms with carcinomatous and sarcomatous features [9,11–13]. Unlike in viral transformation of cultured fibroblasts, MMC-E mouse epithelial cells transformed by Moloney MSV or by IdUrd-induced C3H/MuLV, although anchorage-independent and tumorigenic, were able to retain pericellular matrices composed of fibronectin, laminin, and procollagen types I and III [14].

In the present report we describe cellular polypeptide changes that result from transformation of MMC-E cells with a newly isolated transforming MSV, 3611 [15]. This virus is able to induce rapid morphological transformation of both fibroblastic and epithelial cells in culture and the virus-transformed cells can induce malignant neoplasms in nude mice [15]. We find that the transformed cells, similar to virus-transformed fibroblastic cells, round up and lose their capacity to retain pericellular matrix structures. Collagen production was drastically decreased and the transformed cells produced novel polypeptides with M_r 60,000 and 58,000, which were released into culture medium.

MATERIALS AND METHODS

The New Replication-Defective Transforming Mouse Virus 3611-MSV

The isolation of the virus has been described in an earlier report [15]. In brief, newborn NFS/N mice were inoculated intraperitoneally with an isolate of MuLV obtained by IdUrd activation of methylcholanthere-transformed C3H/10T1/2 cells [16], and six weeks later, were treated with a single dose of butylnitrosourea. The inoculated animals developed lung and peritoneal adenocarcinomas and histiocytic lymphomas. The virus was isolated from cell-free extracts and cocultivated with in vitro propagated NIH/3T3 mouse embryo fibroblasts. By endpoint dilution and transmission of filtered culture fluids to MMC-E Cl 7 mouse epithelial and FRE Cl 3 rat fibroblastic cells and subsequent cloning in soft agar, several nonproductively transformed cell lines were obtained. Superinfection of such clones by various type C virus isolates resulted in the generation of pseudotype virus stocks in which the host range and serologic properties reflected those of helper virus.

Cultures of 3611-MSV-Transformed MMC-E Cells

After infection with 3611-MSV, the MMC-E cells [9] were grown to confluency and seeded in soft agar. Virus-producing cell colonies were isolated. The supernatant fluid from virus-producing transformed cells was infectious and also transformed MMC-E cells as did the virus grown in NIH/3T3 mouse fibroblasts [15]. The colonies of cells isolated from soft agar were morphologically transformed (Fig. 1). Unlike MSV- or C3H/MuLV-transformed cells [14], the association of both virus and nonproducing 3611-MSV-transformed cell colonies to their growth substratum was loose.

Control MMC-E cells and the 3611-MSV transformed cell colonies were cultivated in plastic tissue culture dishes (Falcon Plastics, Oxnard, California) in Eagle

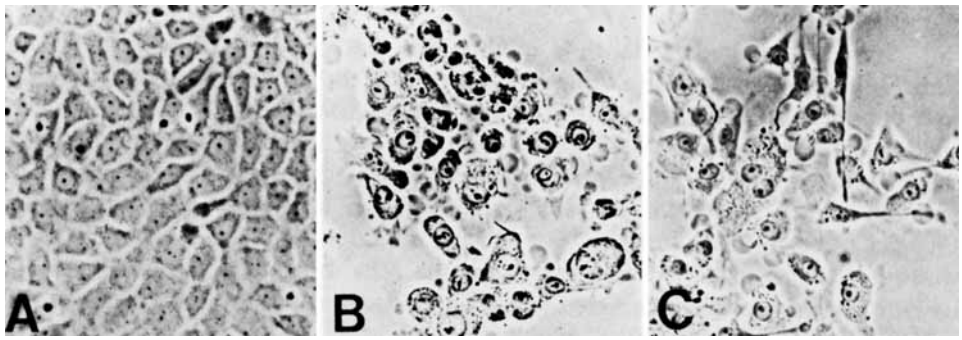

Fig. 1. Phase-contrast photomicrographs of 3611-MSV transformed cells. A) Control MMC-E cells. B) 3611-MSV-transformed virus-producing cells. C) 3611-MSV-transformed nonproducer cells. × 230.

basal medium supplemented with 10% fetal calf serum (Grand Island Biological Co., Grand Island, New York), 2 mM glutamine, 100 IU/ml penicillin, and 50 ug/ml streptomycin. The medium was changed twice a week and the stock cultures of MMC-E cells were subcultured once a week. The 3611-MSV-transformed cells were subcultured every second and third day, otherwise the number of floating cells was drastically increased.

Growth in Soft Agar

The 3611-MSV-infected cells were seeded in 0.3% agar (Difco, Agar Nobel), made up in complete medium containing 10% fetal calf serum; and 2 ml of 0.3% agar was plated on top of 0.5% basal agar. The fraction of cells able to form colonies in soft agar was determined after 1 and 2 weeks of incubation at 37° C. Rapidly growing colonies were selected with a pasteur pipette. The agar piece was transferred to a tissue culture dish, broken into pieces, and the transferred cells were permitted to grow to confluency followed by subsequent subculture.

Metabolic Labeling and Polypeptide Analysis

Cell cultures were labeled with 5 mCi/l (^{14}C)glycine 113 Ci/mol, The Radiochemical Centre, Amersham, England or with 20 mCi/L of both (2-^{3}H)glycine (23 Ci/mmol) and L-(5-^{3}H)proline (15 Ci/mmol, The Radiochemical Centre) in media containing 50 μg/ml of sodium ascorbate and of β-aminopropionitrile fumarate. The radioactive polypeptides of the cell layers and the culture media were analyzed by electrophoresis in sodium dodecyl sulfate (SDS)-polyacrylamide gels [17].

The collagenous polypeptides were identified by their susceptibility to digestion by bacterial collagenase (form III, Advance Biofactures, Lynbrook, New York; 30 U/ml, 60 min at 37°C) and by their electrophoretic mobility when compared to the previously identified collagen polypeptides produced by 3T3 cells [14,18]. The collagenase preparation did not contain contaminating proteinases. The radioactive polypeptides produced by the cells were concentrated by precipitation with ammonium sulfate (final concentration 176 mg/ml) in the presence of proteinase inhibitors and using gelatin as a carrier protein.

Cell Surface Labeling

Surface carbohydrate labeling of the cells was performed by the periodate NaB^3H_4-labeling method [19]. Cells grown on plastic dishes were washed three times with phosphate buffered saline (0.14 M NaCl in 10 mM phosphate buffer, pH 8.0) and incubated with 1 mM NaIO for 10 min on ice washed with phosphate buffered saline, scraped with a rubber policeman and resuspended in 0.5 ml of phosphate buffered saline. Thereafter the cells were incubated with NaB^3H_4 (2 mCi, New England Nuclear, 8 Ci/mmol). After 30 min at room temperature the cells were washed with phosphate buffered saline and the cells were dissolved in gel sample buffer. The samples were analyzed in sodium dodecyl sulphate polyacrylamide gels followed by fluorography [20].

Immunofluorescent Staining of Fibronectin

Human plasma fibronectin was purified as described [21] and the antisera were raised in rabbits. The specificity of the antisera and necessary absorptions have been described in detail in previous reports [18,22]. Fixation of the cells by paraformaldehyde (3.2%) and permeabilization by acetone were performed according to Vaheri et al [22]. The substratum-attached pericellular matrix was isolated according to Hedman et al [23].

RESULTS

Immunofluorescent Staining of Fibronectin

Immunofluorescence analysis for the pericellular fibronectin-containing structures was carried out using paraformaldehyde fixed cell layers and isolated matrices. The isolated matrices of control MMC-E cells contained fibronectin fibers (Fig. 2A), whereas the matrices of 3611-MSV-transformed cells were virtually negative. Analysis of layers of paraformaldehyde-fixed 3611-MSV-transformed cells (surface staining) showed that no fibrillar fibronectin was detectable (Fig. 2B). When the cells were stained after paraformaldehyde and acetone fixation (total staining) cytoplasmic fibronectin was detected, frequently as perinuclear granules (Fig. 2C,D). The 3611-MSV-transformed cells thus contained fibronectin but were unable to deposit it into pericellular matrix structures.

Polypeptide Analysis

The 3611-MSV-transformed cells were labeled with radioactive amino acids and the radiolabeled polypeptides of the culture medium and cell extracts were analyzed by gel electrophoresis followed by autofluorography. Collagenous polypeptides of MMC-E cells were as described earlier [14]. The principal difference between the polypeptides of control MMC-E cells and the 3611-MSV-transformed cells was a pronounced decrease of procollagen secretion (Fig. 3). Virtually no collagenous polypeptides were detected in the culture media of the transformed cells after labeling for 3–6 hr with radioactive amino acids. Longer labeling periods gave evidence that biosynthesis of procollagen was not totally depressed. The deposition of procollagen and fibronectin molecules into the pericellular matrices was impaired. Short pulses with radioactive amino acids labeled fibronectin (M_r 220,000) and also faintly type I procollagen polypeptides. On the other hand, analysis of the substratum-attached molecules of the pericellular matrix preparations indicated that the matrix was lost (gel not shown).

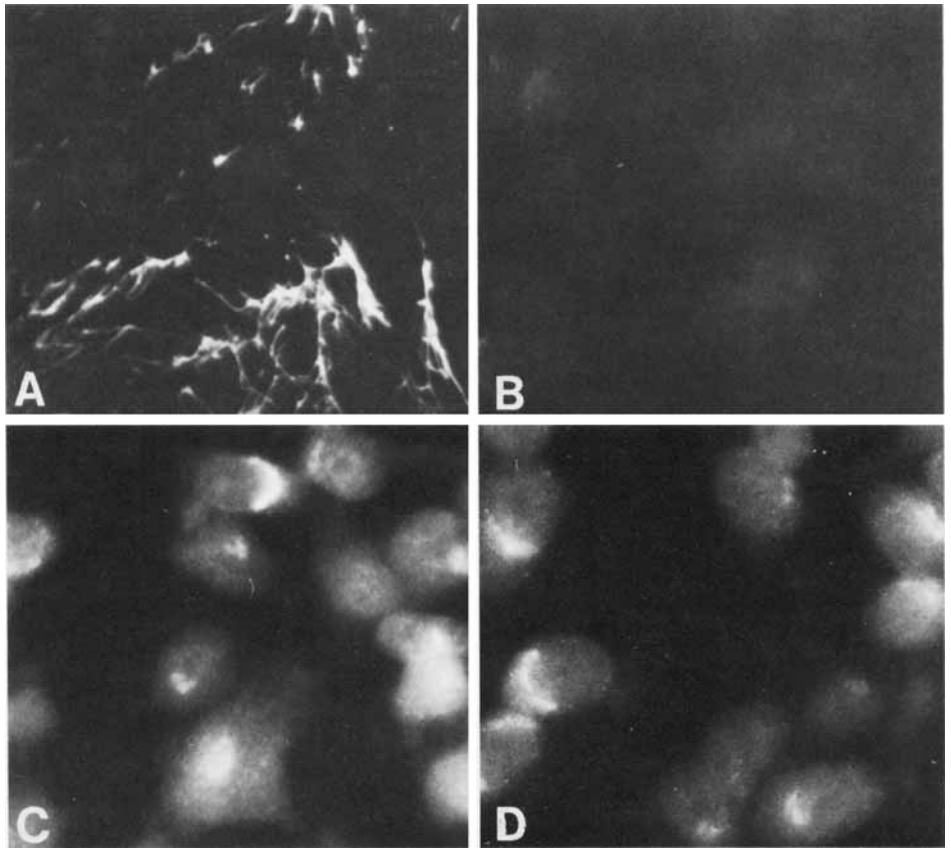

Fig. 2. Immunofluorescence for fibronectin. 3611-MSV-transformed MMC-E cells were grown to confluency and stained with antifibronectin antiserum after fixation with paraformaldehyde (surface staining) or paraformadehyde and acetone (total staining). A) Control MMC-E cells, surface staininig. B) 3611-MSV-transformed nonproducer cells, surface staining. C) 3611-MSV-transformed virus-producing cells, total staining. D)3611-MSV-transformed nonproducer cells, total staining × 520.

Analysis of the polypeptides from the culture medium showed that secretion of two polypeptides migrating as a closely spaced doublet was induced in the transformed cells. Their apparent molecular weights were 60,000 and 58,000 and both of them were labeled with radioactive mannose (Fig. 4). No evidence for precursor product relationship was observed between these two polypeptides. Both of them were also labeled with long pulses of radioactive glucosamine, but the M_r 60,000 polypeptide was, however, preferentially labeled with short pulses of glucosamine. These polypeptides expressed different abilities to bind to heparin-agarose conjugates suggesting that they may be distinct entities (T. Vartio and J. Keski-Oja, unpublished observation).

Cell Surface Carbohydrate Analysis

The transformed cell colonies, producer and nonproducer cells, and control MMC-E cells were labeled by the periodate-sodium borotritide method. Autofluorograms of the gels of the respective samples indicated that a M_r 100,000 glycoprotein

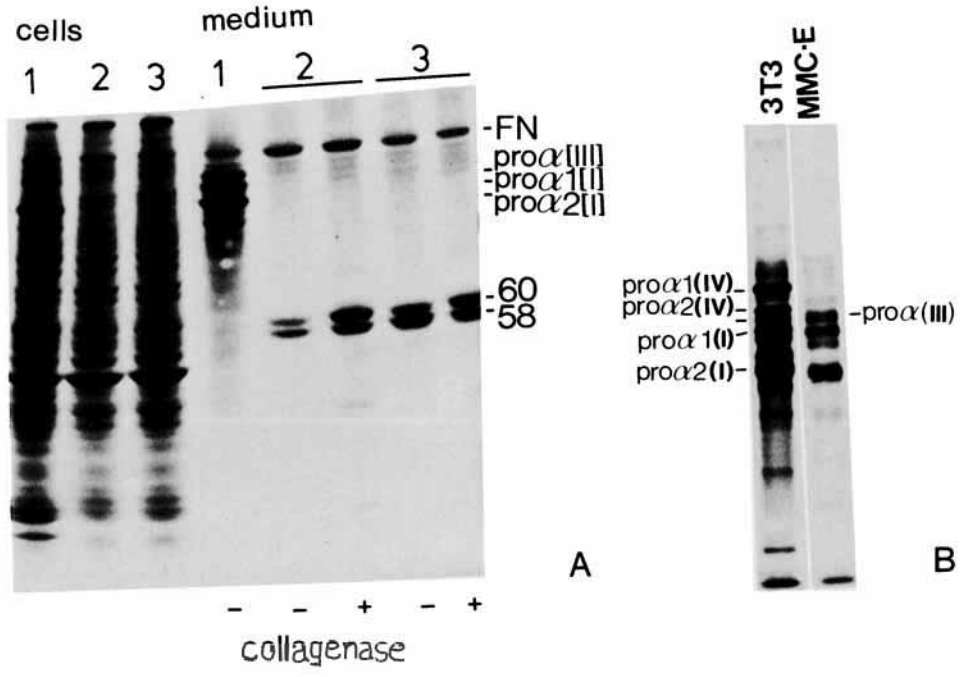

Fig. 3. Polypeptide analysis of 3611-MSV-transformed cells. A) The cells were labeled with ^{3}H-glycine and ^{3}H-proline for 5 hr in the presence of ascorbate (see Methods) and the radiolabeled polypeptides of the cell layers and medium were analyzed in a 5–16% gradient polyacrylamide gel followed by autofluorography. 1)MMC-E cells. 2)3611-MSV nonproducer cells. 3) 3611-MSV producer cells. Collagenase digestion is indicated. The migration of fibronectin (FN) and the chains of procollagen type I and III are indicated. The position of the 58,000- and 60,000-dalton polypeptides are shown. B) Comparison of the polypeptides produced by 3T3 fibroblasts and MMC-E cells in a 5% polyacrylamide gel. The mobilities of procollagen type I, III and IV chains are indicated.

was expressed both at the surface of the producer and nonproducer cells. In addition, a M_r 68,000 glycoprotein was seen in both virus-transformed cell colonies (Fig. 5). These glycoproteins were clearly separate from the M_r 60,000 and 58,000 polypeptides.

DISCUSSION

The present results indicate that transformation of mouse epithelial MMC-E cells by 3611-MSV affects several mechanisms that operate in uninfected cells. The following major changes were observed: (1) the pericellular fibronectin matrix is lost, (2) the biosynthesis of procollagen is reduced, (3) two major glycoproteins with M_r 100,000 and 68,000 are expressed at the cell surface, and (4) the secretion of polypeptides with M_r 58,000 and 60,000 is initiated.

Carcinomas are the most common neoplasms of humans and the biology of carcinoma cells differs considerably from that of sarcoma cells [cf 24,25]. Viral transformation of nontumorigenic epithelial cells in culture has been difficult to study because of the lack of suitable model cultures. Rat liver epithelial cells are easily

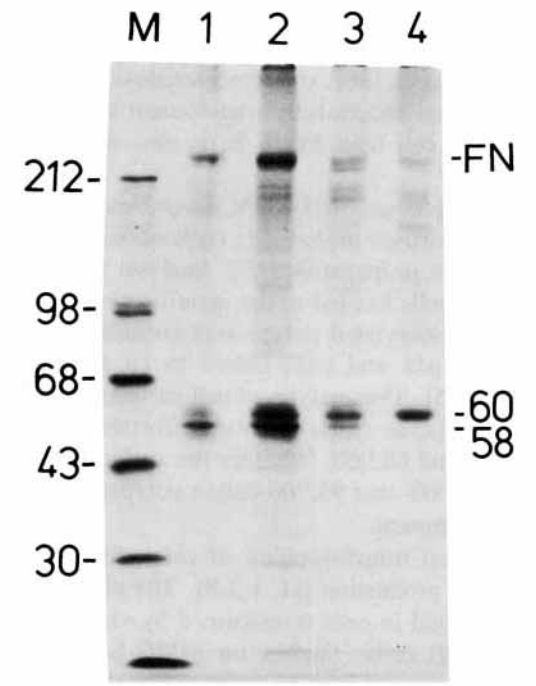

Fig. 4. Metabolic carbohydrate labeling of the polypeptides produced by 3611-MSV-transformed cells. The cells were labeled with ^{3}H-glycine and ^{3}H-proline (lanes 1,2) or with ^{3}H-mannose (3) or ^{14}C-glucosamine (4). M-molecular weight markers. The labeling times were (1) labeling for 4 hr, (2) 8 hr, (3) 4 hr, (4) 4 hr. Note that the 60,000- and 58,000-dalton polypeptides are not labeled concomitantly.

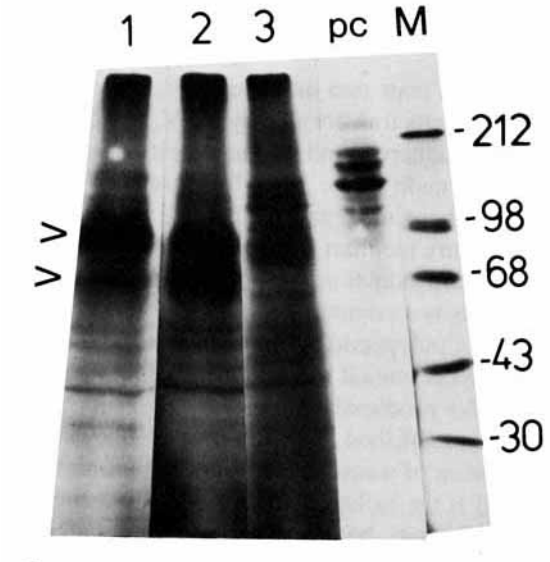

Fig. 5. Cell-surface carbohydrate labeling of 3611-MSV-transformed cells. Cells grown to confluency were labeled by the periodate $NaB^{3}H_4$ method followed by gel electrophoresis and autofluorography. 1)3611-MSV nonproducer cells. 2) 3611-MSV producer cells. 3) Control MMC-E cells. pc-procollagen markers; M-molecular weight markers ($\times 10^{-3}$). Arrowheads indicate the position of the 100,000- and 68,000-dalton polypeptides.

available and they have been used in a number of studies [26–29], but because of their origin they probably do not reflect well the natural situation in carcinogenesis. Cultures of adrenal cortex cells [30], ovarian granulosa cells [31], and rat thyroid epithelial cells [32] have been successfully transformed with type C viruses. Nontumorigenic mouse epithelial cell line, MMC-E, is also susceptible to infection with type C viruses [9,11,15].

The acute transforming virus, 3611-MSV, resembles previously described mammalian acute transforming viruses in that it is replication-defective, requiring a type C helper virus for successful propagation [15]. Analysis for viral antigen expression in 3611-MSV-transformed cells has led to the identification of a M_r 90,000 polyprotein and a M_r 75,000 phosphorylated polyprotein consisting of amino acid terminal MuLV gag gene proteins p15 and p12, linked to an acquired sequence-encoded nonstructural component [15]. The analysis of cell surface carbohydrates showed that 3611-MSV-transformed cells, as C3H/MuLV-transformed cells [14] expressed glycoproteins of M_r 100,000 and 68,000. Whether the surface-labeled and immunologically detected [15] M_r 100,000- and 90,000-dalton polypeptides have any relationship to each other is unclear at present.

It has been shown that transformation of chick fibroblasts by RSV causes a major decrease in collagen production [cf, 1,2,8]. The phenotype of type I procollagen is also frequently affected in cells transformed by viruses and chemical carcinogens [33], also in MMC-E cells. Studies on MMC-E cultures transformed with Moloney MSV and ecotropic C3H/MuLV revealed that the deposition of procollagen type I was affected so that procollagen type I was preferentially incorporated in the cell layers as procollagen a α_1-(I)-trimers [14]. These cells, however, continued to produce also normal type I procollagen into their medium. In ethylnitrosourea-transformed cells, on the other hand, the production of collagen was affected and no procollagen α_2-(I) chains were observed [12]. Malignant transformation of mouse epithelial cells thus commonly affects procollagen production and deposition, but the mechanisms evidently vary.

There are probably at least two different ways in which MMC-E cells can be malignantly transformed. Cells transformed by MSV, C3H/MuLV, and by ethylnitrosourea [12] remained well adherent and retained their pericellular matrices, although they were tumorigenic in nude mice. Tests for basement membrane collagenolytic activity have shown that these malignant cells have achieved the ability to produce this enzyme into their culture medium as a result of transformation [34].

The expression and production of polypeptides with molecular weights of 53,000 and 56,000 daltons is a common feature of different malignantly transformed cells [35–38]. This kind of polypeptide has been observed also recently using rat cells transformed with a T-class *ts* mutant of RSV [8]. The relationship of the 58,000- and 60,000-dalton polypeptides produced by 3611-MSV transformed MMC-E cells to the other ones remains to be established.

So far, the mechanism of transformation has not been elucidated in any system. What has been achieved is the isolation of cell-derived oncogenes as part of retroviruses or by DNA transfection; both procedures are apparently yielding the same family of cellular genes [39]. Virus-transduced oncogenes can be classified according to the biological activity of their tumorgene products into those that code for a protein kinase with specificity for tyrosine in certain cellular peptides and those that do not have this activity. Thus, there may be two different pathways of transformation being

taken as cells become transformed by one or the other class of cellular or cell derived tumorgenes. An independent line of experimentation has pointed to the possibility that polypeptide growth factors may be involved in the maintenance of the transformed state. One class of these growth factors that are turned on in transformed fibroblasts upon transformation with a variety of mammalian tumorgene-transducing retroviruses are related to epidermal growth factor [40] and share with it the capacity, together with another less well-characterized peptide, to induce cells to anchorage independent growth. Growth factor-mediated transformation, which occurs much more readily with fibroblastic than with epithelial cells, may or may not utilize an intracellular pathway involving the activation of tyrosine specific protein kinase [39]. It will now be interesting to determine the relationship of the 58,000- and 60,000-dalton peptides to either growth factors or growth factor-dependent transforming factors and whether the transformed epithelial cells release activity which preferentially transforms phenotypically epithelial cells.

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