SV40-Transformed Hamster Lens Epithelial Cells: A Novel System for the Isolation of Cytoskeletal Messenger RNAs and their Translation Products

H. Bloemendal,* J. A. Lenstra,* H. Dodemont,* F. C. S. Ramakers,* A. A. Groeneweld,* I. Dumia† and E. L. Benedetti†

*Department of Biochemistry, University of Nijmegen, Geert Grooteplein N 21, 6525 EZ Nijmegen, Netherlands, and †Institute de Biologie,§ Moléculaire du CNRS§ et de l’Université, Paris VII, 2 Place Jussieu, Paris, France

(Received 20 May 1980, New York)

Epithelial cells from Syrian hamster lens were cultured and transformed by Simian Virus 40 (SV40). This cell line can be brought into suspension culture and then grows very efficiently. The quantitatively major proteins synthesized by these cells appeared to be the cytoskeletal constituents actin and vimentin. Morphologically the occurrence of the two proteins in the cultured cells is reflected by the presence of actin filaments and intermediate-sized filaments. This was demonstrated by immunofluorescence studies and electron microscopy. A messenger RNA fraction that was isolated from the transformed cells directed almost exclusively the synthesis of actin and vimentin after incubation in a heterologous cell-free system.

Key words: transformed lens cells; cytoskeletal mRNA; lens suspension culture

1. Introduction

The lens epithelium constitutes a pure and well-defined cell population. Mammalian lens epithelial cells from various species can be cultured for extended periods of time (Van Venrooij, Groeneweld and Benedetti, 1974a, b; Courtois, Simonneau, Tassin, Laurent and Malaise, 1978; Tassin, Malaise and Courtois, 1980). All studies reported so far in the literature made use either of explants or monolayer cell cultures. Some years ago Albert, Rabson, Grimes and Von Sallmann (1969) described a hamster lens cell line that could be transformed by SV40. These workers observed the formation of multiple layers and clumps of cells. We wondered whether this system could be explored by developing a suspension culture and if so which were the major proteins manufactured by the transformed cells.

We wish to report here that our attempts were successful in that indeed a suspension culture could be obtained. Moreover, the cytoskeletal proteins actin and vimentin were synthesized in appreciable amounts in agreement with the morphological visualization of actin filaments and intermediate-sized filaments. On the other hand, crystallin biosynthesis had either stopped or reached a level that was below the limits of detection, as previously reported for cultured cell lens cells (Van Venrooij et al., 1974a).

The present paper describes some of the features of the suspension culture, the isolation of cytoskeletal messenger RNA's and the characterization of their translation products.

2. Materials and Methods

Establishment of a hamster lens epithelial cell line.

Lenses from 8-week-old Syrian gold hamsters were rinsed with 0.38% trypsin in Ca²⁺- and Mg²⁺-free Tyrode solution in order to remove adhering material. The tissue was divided over two Falcon flasks (bottom: 25 cm²), minced with scissors, partially dried for 15 min in order to promote adhesion and incubated at 37°C in 5 ml of a medium derived from the following stock

solution: 1.5 g lactalbumin hydrolysate dissolved in 800 ml double-distilled water supplemented with 30 ml sterile Hank's A and 30 ml Hank's B solution, both concentrated × 20 (Medium A). 3.24 g TE199 powder medium, dissolved in 40 ml double-distilled water and filtered through a 0.2 μm Millipore filter (Medium B), is added to Medium A. Shortly before use, calf serum, 100 μg/ml penicillin, 100 μg/ml streptomycin and 20 ml of a 0.2 mM-glutamine solution is added. Firstly a monolayer culture is obtained which is maintained as reported previously for cultured calf lens epithelium (Van Venrooy et al., 1974a). Transformation was achieved by incubation with 5 ml of a JL SV40 suspension (kindly provided by Dr. A. Berns from our laboratory) in the presence of 5 μg/ml polybrene. The titer of the virus was 10⁹/ml, measured in an XC-cell plaque assay.

The transformed cell line was brought into suspension in Eagle's minimal essential suspension medium, supplemented with 20% fetal calf serum and vitamins, and maintained at densities from 0.5 × 10⁴ to 1.5 × 10⁶. Cell division occurred every 15–20 hr.

Tests of malignant transformation

The capability of forming colonies in methylcellulose gels was assayed after incubation of trypsinized cells in 3 ml 0.7% (w/v) methocel MC4000 CP (Fluka AG, Buchs, Switzerland), 0.4% (w/v) NaHCO₃ in growth medium at 37°C. Oncogenicity was tested in nude mice by subcutaneous injection of 10⁶ cells (monolayers) or 10⁵ cells (suspension) in 0.5 ml Tyrode solution. The animals (strain B10 LP nu/nu) were obtained from TNO, Zeist, The Netherlands.

Immunofluorescence

The procedure is identical to that applied for cultured calf lens and described elsewhere in full detail (Ramaelers, Osborn, Schmidt, Weber, Bloemendal and Franke, 1980). In the present study monospecific antisera directed against phophatidylserine, actin and vimentin have been used. Cells from the suspension culture were sedimented onto glass slides before applying the antibodies.

Immunoprecipitation

SV40 tumor antiserum was a kind gift of Dr. P. L. Schrier, State University of Leyden. Immunoprecipitation was carried out as described by Schrier, Van der Eise, Hartoghs and Van der Eb (1979).

Isolation and characterization of mRNA

Lens polyribosomes were isolated as described by Bloemendal, Schoenmakers, Zweers, Maas and Benedetti (1986). After suspending, the polysomal pellet samples were subjected to affinity chromatography on oligo-dT-cellulose columns as reported by Vermorken, Hildrink, Van de Ven and Bloemendal (1976). The RNA fractions obtained were treated with methylmercury-hydroxide (Dowonnet, to be published) and centrifuged through an isokinetic sucrose density gradient (15–35%). Both the poly A(−) and poly A(+) fractions were translated in an RNA-dependent reticulocyte cell-free system as described by Pelham and Jackson (1976).

Gel electrophoresis

Sodium dodecylsulphate polyacrylamide gel electrophoresis (13% or 7–18% acrylamide) was carried out as described by Laemmli (1970). Two-dimensional gel electrophoresis was performed as described by O'Farrell (1975). Gels were stained and destained as described by Weber and Osborn (1966). Radioactive bands and spots were detected by fluorography as reported by Bonner and Laskey (1974).

Electron microscopy

Cells from the suspension culture were gently centrifuged at 800 × g for 5 min and washed with the culture medium. 3.5% glutaraldehyde in phosphate buffer was added to the cells resuspended in the culture medium. After 15 min of prefixation the cells were sedimented again and fixed for 1 hr in the same buffered glutaraldehyde containing 1% of galloyl glucose. Thereafter the cells were repeatedly washed and postfixed in 1% osmium tetroxide dissolved in the phosphate
buffer. Then the cells were dehydrated and embedded in a mixture of Epon–Araldite. Thin sections were stained with uranyl acetate and lead citrate.
Electron microscopic observations were made in a Philips EM 400.

**Impulse cytophotometry**

In order to determine their relative DNA content, the cells were analyzed by impulse cytophotometry measuring the amount of ethidium bromide intercalated to the nuclear DNA. The procedure was essentially as described by Dittrich and Göhde (1969).

3. Results

**The suspension culture of hamster lens cells**

After about 7 days of growth of the primary culture the cells were infected with JL SV40 virus. The transformed cells see [Fig. 1(b)] were subcultured several times, in order to get sufficient material for the suspension culture, trypsinized and transmitted into the suspension medium as described in the method section.

Already after 24 hr strong growth was observed. Some heterogeneity of the DNA content per cell was observed as judged from the impulse cytophotometric analysis (Fig. 2), which is consistent with the aneuploid character of transformed cells in general. When the cells were grown in suspension this feature was maintained.

**Immunofluorescence studies**

Figure 3 shows the distribution of actin filaments (a, b, c) and intermediate-sized filaments (d, e, f) both in normal and transformed hamster epithelial lens cells grown in monolayers and in suspension culture. The filaments are visualized using the indirect immunofluorescence method with antibodies directed against actin and vimentin, respectively. It is striking that lens epithelium contains the mesenchymal type of intermediate-sized filaments with vimentin as protein subunit (Ramaekers et al., 1980) rather than prekeratin or as has been found a combination of the two types of motility protein (Osborn, Franke and Weber, 1980, in other epithelial cells, Lazarides, 1980). Prekeratin could not however, be demonstrated in cultured lens cells, irrespective of whether the cells were derived from calf or hamster lens.

The typical immunofluorescence pattern of the intermediate-filament structure is seen in normal and transformed monolayer culture cells as well as in the transformed cells derived from the suspension culture. Apparently the actin organization has changed in the suspension culture as can be concluded from the diffuse immunofluorescence pattern obtained with anti-actin serum [Fig. 5(c)], and has been substantiated by electron microscopy.

**Electron microscopy**

Electronmicroscopic observation of the cells growing in suspension reveals that the cellular elements are joined loosely together in a small group. The cell–cell interaction is ensured by intermingled digitations and microvilli. Moreover, small junctional regions are also visible mostly of the type of maculae adherens or intermediate junctions. Gap junctions are almost absent. Freeze fracturing studies still in progress will elucidate whether both gap and tight junctions participate in the cell-to-cell attachment and communication.

The general organization of the cytoplasm is characterized by a relatively poorly
developed rough endoplasmic reticulum (ER). This structure is represented by short regions of rather large cisternae filled with amorphous electron-dense material. Tangential sections through the ER membrane profile show that the ribosomes have spiral shape [Fig. 4(a)]. On the other hand most of the ribosomal material is largely scattered over the whole cytoplasm from the perinuclear region up to the cell periphery. In the intact cell the cytoskeleton organization is hardly detectable. However, intermediate-sized filaments are clearly visualized around the nuclei and spread all over the cytoplasm forming loose bundles. Microtubules as well are visible without any particular distribution, being mostly randomly scattered. The detection of the pattern of thin actin filaments is very difficult. The most evident feature is that in many cells, but not in all, a rather
compact filamentous meshwork is found in close proximity to the plasma membranes or comprised within the core of cell surface microvilli. In other cytoplasmic regions a thin filament meshwork is found in areas filled by polyribosomes. The nuclear structure will not be described here in full detail.

If the cells from the suspension culture are gently extracted by Triton X100 (Ben-Ze'ev, Duerr, Solomon and Penman, 1979) then the abundance of intermediate-sized filaments (IF) is visualized. In fact from the nuclear envelope up to the plasma membrane an intricate meshwork of IF is seen. We found in cells obtained after several subculturing steps that in contrast to the very large amount of polyribosomes visualized prior to Triton extraction, after this treatment the bulk of ribosomes was removed [compare Fig. 4(a) with 4(b), (c)]. Virtually only a few clusters of polyribosomes remain attached either to membrane profiles or to remnants of a tiny filamentous meshwork [Fig. 4(c)]. This observation is sustained by negative staining which allowed the visualization of the characteristic structure of IF [Fig. 4(d)] in contrast to minor amounts of rather short 7 nm actin filaments.

Protein pattern of transformed lens cells

Figure 5(a) shows the 2D-electrophoretic pattern of proteins extracted from the hamster lens cells in suspension culture. The most predominant spot can be identified as vimentin (Ramaekers et al., 1980) followed in intensity by actin. All other, hitherto unidentified, spots are less heavily stained. That SV40 DNA is incorporated into the hamster lens cell genome could be proven by immunoprecipitation with SV40 tumor antiserum [Fig. 5(b)]. Both the transformed monolayer cells and SV40 transformed rat cells which were used as control, showed specific immunoprecipitation of the SV40 T-
FIG. 3. Immunofluorescence microscopy of cultured hamster lens cells. (a, d) normal monolayer; (b, e) transformed monolayer; (c, f) transformed suspension culture; (a, b, c) incubation with antiserum directed against actin; (d, e, f) incubation with antiserum directed against vimentin. (Bars indicate 10 μm.)
antigen and a 55000 dalton protein, presumably the cell-coded SV40 middle T-antigen (Rigby, 1973). The control shows also a very weak band in the 55000 mol. wt. region. Definite proof that this component differs from the middle T-antigen awaits further experiments.

Cytoskeletal messengers and their translation products

Polyribosomes were isolated from the suspension culture as described in the method section. After fractionation by affinity chromatography on oligo-dT-cellulose columns they were further separated by sucrose density centrifugation [Fig. 6(a)]. The 16–18S region from the gradient was collected and translated in a reticulocyte cell-free system. It can be seen from the 2D-electropherogram that this fraction in particular directs the synthesis of actin and vimentin [Fig. 6(b)]. Furthermore a hitherto unidentified, rather strong, spot is observed in the 48 K range. This protein is, according to its location on the focusing gel (first dimension), much more basic than actin and vimentin. The same component can also be seen in the pattern of protein extracted from the cultured cells as a spot of intermediate intensity [compare Fig. 5(a)].

4. Discussion

Epithelial lens cells from Syrian hamster undergo transformation in vitro after infection with Simian Virus 40 (Albert et al., 1969). It appeared from our experiments that such cells can be cultured in suspension high amounts of transformed lens cells. We conclude that the viral genome is incorporated into the host DNA as the protein distribution pattern on SDS-gels revealed the presence of the T-antigen after transformation. Albert et al. (1969) demonstrated that the transformed cells produced tumors when injected into homologous hosts. Courtois et al. (1978) made the striking observation that adult cattle lens cells in long-term culture acquire spontaneously some characteristics of transformation. For instance, upon injection into nude mice those cells provoke tumors which synthesize α-crystallin. In contrast, cells from our suspension culture did produce tumors in nude mice but we were unable to detect the synthesis of crystallin polypeptides either in the culture or in the tumor. The major proteins manufactured by the cultured cells are the cytoskeletal components actin and vimentin. The corresponding messengers could easily be obtained from polysomes isolated from the suspension culture. Apparently these mRNA's exist in a poly A+ [see Fig. 6(a)] and a poly A− form (translation pattern not shown). Whether the presence of this poly A−mRNA is due to spontaneous degradation or rather represents the result of any post-transcriptional event cannot be decided yet. Likewise the process by which vimentin and actin production is regulated in normal and transformed lens cells in vitro remains to be elucidated.

The motility proteins actin and vimentin which contribute to the formation of a well-developed cytoskeleton have been visualized both by immunofluorescence and electron microscopy. Here a full account of the differences in cytoskeletal organization between cells in monolayer and in suspension culture has not been given but we would like to summarize briefly some main points.

The stable filamentous organization of actin and vimentin is enhanced upon attachment of the cells to a substrate. Experiments to be published elsewhere provide evidence that Triton X100 ghosts derived from the cells in suspension culture contain vimentin-IF as major filamentous component and very few actin filaments. The latter filaments appear to be associated with ribosomal clusters. Experimental evidence supports the
Fig. 4. Electron micrographs of SV40-transformed hamster lens cells in suspension culture. (a) The cytoplasm close to the nucleus is filled by a great number of polyribosomes. Some spiral polyribosomes (arrow) are attached to the membrane. Bundles of intermediate-sized filaments are running in various directions. (Thin sections stained with uranyl acetate and lead citrate.) (b) Same cells as in (a) subjected to Triton X-100 treatment. Thin 7 nm filaments are visible together with few intermediate-sized filaments. Note that polyribosomal clusters remain attached either to membrane profiles or to the thin filaments (arrow). (Thin section stained with uranyl acetate and lead citrate.) (c) Cells treated as in (b). From the nuclear envelope to the plasma membrane bundles of intermediate-sized filaments are visible. Among them, few rough membrane profiles and a meshwork (arrow) of tiny filaments with few polyribosomes attached are visible. (Thin sections stained with uranyl acetate and lead citrate.) (d) Same cells as in (b). The cellular ghost has been spread onto a carbon film and negatively stained with uranyl acetate. A number of intermediate-sized filaments are clearly visible in close proximity to membrane sheets (M).
Fig. 5. (a) 2D-electrophoretic pattern of protein extracted with SDS from hamster lens cells cultured in suspension. A, actin; V, vimentin. The two proteins were identified by co-migration on 2-D gels with the purified lens actin and vimentin, respectively. (b) Fluorogram of a polyacrylamide gel (7-18%) electrophoretic pattern of immunoprecipitates obtained after addition of SV40 antiserum to lysates of (a) normal monolayer, (b) transformed monolayer, and (c) SV40-transformed baby rat kidney cells. Numbers indicate the molecular weights of marker proteins (d). The asterisks indicate the SV40 T-antigen and the 55K middle T-antigen. The faint bands at the bottom of the gel may represent the SV40 T-antigen. 200000 = myosin; 93000 = phosphorylase-b; 68000 = bovine serum albumin; 45000 = ovalbumin; 30000 = carbonic anhydrase.
assumption that deletion of a fully organized cytoskeleton parallels cell transformation. This phenomenon has been assessed at least in studies upon cell transformation induced by DNA or RNA virus (Goldman, Yerex and Schloss, 1976; Paulin, Nicolas, Yaniv, Jacob, Weber and Osborn, 1978). Other data, however, show that this conclusion is not a general rule (Watt, Harris, Weber and Osborn, 1978). Actually loss of the feature of transformation as a result of growing at non-permissive temperature is followed by reappearance of a fully developed cytoskeleton. Our results suggest that changes in the cytoskeletal organization are dependent rather upon growth conditions in particular whether or not the cells are growing in suspension or attached to a substrate. Since several authors pointed out the role of fibronectin with regard to cell attachment,
cytoskeletal assembly and transformation (Singer, 1979; Hynes, Destree, Perkins and Wagner, 1979), further studies also in this line are in progress in our laboratories. Preliminary results using antibodies directed against fibronectin indicate that the latter glycoprotein is still present at the cell surface of transformed in cells monolayer.

ACKNOWLEDGMENTS

The authors are grateful to Dru M. Osborn and K. Weber (Max Planck Institute for Biophysical Chemistry, Göttingen) and to Dr W. Franke (German Cancer Research Center, Heidelberg) for their kind gift of antibodies directed against actin and vimentin.

These investigations were partly carried out under the auspices of the Netherlands Foundation for Chemical Research (SON) and with financial aid from the Netherlands Organization for the Advancement of Pure Research (ZWO). H. B. is recipient of a North Atlantic Treaty Organization grant.

REFERENCES


