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Inhibition of Chicken Embryo Lens Differentiation and Lens Junction Formation in Culture by pp60$^{v\text{-}src}$

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A culture system was developed which permitted the differentiation of chicken lens epithelial cells to lentoid bodies which contained several cell layers, accumulated high levels of delta-crystallin, and produced extensive gap junctions. This differentiation process was prevented when the cells were infected with a temperature-sensitive *src* mutant of Rous sarcoma virus and maintained at the permissive temperature. These transformed cells continued to proliferate and also synthesized the major lens gap junction protein, MP28, at near-normal rates. However, this MP28 was not assembled to produce gap junctions. Cultures shifted to the nonpermissive temperature formed lentoid bodies similar to those in uninfected lens cultures, including the establishment of gap junctions containing MP28.

The effect of the expression of the v-*src* gene associated with Rous sarcoma virus (RSV) on the differentiated state of infected cells is specific to the cell type involved. In myoblasts (11, 14, 22), chondroblasts (1, 23), and retinal melanoblasts (7, 26) the expression of differentiated cell functions is suppressed. In contrast, v-*src* has no effect on the differentiation of macrophages (10) and stimulates the differentiation of PC12 cells as measured by neurite extension (2). From the diversity of effects it is clear that the chain of events within the cell initiated by an active v-*src* product depends on the particular cell type involved. The molecular mechanism behind the suppression of synthesis of differentiated cell products by v-*src* has been studied in the most detail in the chondroblast system. The major products of chondrogenic differentiation are the components of their extracellular matrix. The synthesis of these components is coordinately suppressed by v-*src* and appears to be regulated primarily at the mRNA level (1, 3). In addition to the suppression of synthesis, the expression of v-*src* may affect morphological differentiation by other mechanisms. The lens culture system provides some unique features for analysis of these effects.

Chicken embryo lens cells cultured in vitro under the proper conditions proliferate to produce an epithelial monolayer which proceeds to the morphogenesis of multicellular, multilayered, lentoid bodies which resemble normal lens structures. Within the lentoid bodies up to 50% of the cell membrane contains junctions, the primary vehicle for intercellular communication. Since the v-*src* protein is membrane associated and the major gap junction protein MP28 is a phosphoprotein (15), it is possible that the function or assembly of the junctions could be directly regulated by the action of the v-*src* kinase.

The effect of oncogenic transformation on permeable junctions has long been a matter of debate. However, the basic hypothesis for many years has been that blockage of the gap junctions results in cellular deregulation (17). Although this interpretation was questioned in early studies, more recent studies with Rous sarcoma virus and temperature-sensitive v-*src* mutants have demonstrated that there is a substantially reduced intercellular permeability at the permissive temperature for transformation (4, 5). Another study demonstrates that when functional communication can be established between normal and transformed cells via gap junctions, growth inhibition of the transformed cells occurs (18). The limitation of the previous studies on gap junctions is that cell lines were used in which only a small proportion of the membrane is involved in the gap junctions. The lens cultures not only accumulate a large percentage of junctions in the membrane but also exhibit a number of differentiated features characteristic of the lens (20). The high percentage of junctional membrane in the lens cultures, far in excess of what has been reported for other cell types, allows a straightforward analysis of the effect of transformation on synthesis of the major junctional protein and on junction formation. The junctions which have been described in the lens resemble gap junctions that are found in other tissues (6, 12). Electrophysiological studies (25) and dye transfer experiments (28) suggest that these lens junctions contain cell-to-cell channels. Much of the literature supports the view that the major lens membrane protein, MP28, is a major component of the lens junctions (8, 27), although some investigators have evidence to the contrary (24). Recent studies which show that the microinjection of antibody to MP28 blocks dye transfer in differentiating lens cultures (K. Klukas, E. Hertzberg, D. Spray, and R. G. Johnson, J. Cell Biol. **105**:227a, 1987) strongly supports the view that MP28 is a major component of the lens junction. In this study on the lens culture system, evidence is presented that shows that junction formation, but not synthesis of the major junctional protein, is inhibited by pp60$^{v\text{-}src}$.

MATERIALS AND METHODS

Cells and viruses. Chicken embryo lens cultures were prepared as described by Menko et al. (20). Cells were dispersed by incubation in trypsin accompanied by mechanical agitation. After being passed through a lens paper filter to obtain single cells, the cells were routinely plated at densities of 0.5×10^6 to 1×10^6 cells per 35-mm petri dish (1×10^5 to 2×10^5 cells per cm^2). For control cultures the dishes were collagen coated as previously described (20), but it was not necessary to collagen coat the dishes for growing transformed cells. At 2 to 4 h after plating, the cells were infected with RSV at a multiplicity of infection of 4 focus-forming units per cell with either strain SRD or tsLA24A, a

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mutant of RSV which is temperature sensitive for pp60$^{v\text{-}src}$. The infected cells were passaged two to four times to ensure complete infection by the transforming virus.

Antiserum. The rabbit antiserum to the MP28 lens junctional protein was made against purified MP28 prepared as follows. Chicken lens junctional membranes were purified through the first sucrose gradient step of Goodenough (12). The major protein component, which migrated at 28 kilodaltons on a sodium dodecyl sulfate-polyacrylamide gel, was cut out and further purified by electrophoresis through sodium dodecyl sulfate-polyacrylamide gels three times. The final acrylamide band was used as the source of the antigen. The complete preparation and characterization of this antibody have been described previously (20). When used in immunofluorescence studies of thick sections of 10-day chicken embryo lenses, the antibody stained the membranes of all but the epithelial cells, with staining intensity increasing toward the nuclear core of the lens. In cultures of chicken embryo lens cells the antibody stained cell membranes as early in differentiation as the establishment of the epithelial monolayer with cobblestone-like packing.

Immunofluorescence. Cells prepared for immunofluorescence were plated on Lab-Tek slides (no. 4808) at a density of 4.5×10^4 cells per well. The cells were washed in phosphate-buffered saline, pH 7.3 (PBS), fixed in 3.8% formaldehyde (8 min, 22°C), washed, postfixed in acetone (−20°C, 5 min), and replaced in PBS to keep the cells from dehydrating. The cells were incubated first with calf serum (diluted 1:10 in PBS; 37°C, 1 h) to eliminate nonspecific binding and then washed in PBS. Then the cells were incubated with the anti-MP28 serum (1 h, 37°C), rinsed in several changes of PBS, and then incubated with rhodamine-conjugated goat anti-rabbit immunoglobulin G (1 h, 37°C). The cells were washed extensively in PBS followed by distilled water, mounted in elvanol, and observed in a Zeiss microscope with epifluorescence.

Thin sections. Chicken embryo lens cultures were rinsed in medium and fixed with 2.5% glutaraldehyde in 0.1 M cacodylate (pH 7.3) for 1 h at room temperature. Cells were rinsed again, postfixed with osmium tetroxide, stained with uranyl acetate (1% in maleate buffer) for 1 h, dehydrated through a graded series of ethanol, infiltrated overnight at room temperature with Epon 812, and then embedded in Epon 812. The culture dish was pried away before sectioning. Thin sections were observed in a Hitachi 600 electron microscope at 75 kV.

Protein labeling. Chicken embryo lens cells were grown in 35-mm tissue culture dishes, rinsed with TD buffer (0.14 M NaCl, 0.005 M KCl, 0.0007 M Na_2PO_4, 0.025 M Tris base, 5 mM D-glucose; pH 7.3), and labeled in 100 μl of Dulbecco modified Eagle medium with 5% fetal calf serum containing 50 μCi of [^{35}S]methionine per ml. Incubation was for 2 h at 36°C. Cells were washed and harvested directly in 200 μl of electrophoresis sample buffer (0.06 M Tris [pH 6.8], 2% sodium dodecyl sulfate, 10% glycerol, 5% mercaptoethanol, 0.002% bromophenol blue).

Electrophoretic analysis of proteins. Electrophoresis was carried out with Laemmli 10% polyacrylamide slab gels (16). Lens cultures were labeled with [^{35}S]methionine and solubilized in sample buffer for 10 min at 95°C. Total radioactive counts were determined for each sample, and identical counts were applied to each well. Marker samples for molecular size determination included standard molecular size markers bovine serum albumin, ovalbumin, pepsin, trypsinogen, beta-lactoglobulin, and lysozyme (range, 14.3 to 68 kilodaltons). The gels were stained with Coomassie blue, destained, and then treated with Autofluor (National Diagnostics), an autoradiographic image intensifier. Autoradiographs were scanned in a Zeineh soft laser scanner densitometer.

RESULTS

Inhibition of lens cell differentiation by RSV. Lens cells begin differentiating after only a short time in culture (20). Therefore, they were routinely infected with the *src* virus within 2 to 4 h after plating as primary cultures. Early after infection these lens cultures (Fig. 1c) still appeared morphologically similar to control cultures (Fig. 1a), but there was a significant increase in cell number. This increase in cell number may be an early effect of transformation or the result of other stimulatory activity in the virus inoculum. By 6 days after infection transformation of these cells was widespread (Fig. 1d). At this point in a normal culture, lens cell differentiation, which is characterized by lentoid formation, is essentially complete (Fig. 1b). These lentoids are complex multicellular, multilayered structures as described in previous studies of cross-sections at both the light and electron microscopic levels (20). The transformation of lens cultures with a v-*src*-containing virus resulted in the inhibition of lens cell differentiation. The cells in these transformed cultures were characterized by an increased refractility, and the cultures contained many areas of piled up rounded cells, both typical of RSV transformation. Those cells in the culture which had not become infected proceeded through the normal stages of differentiation. Therefore, for all experimental procedures the cells were passaged and used as either secondary or tertiary cultures. These passaged cultures of RSV-infected lens cells consisted exclusively of transformed lens cells; morphological differentiation was completely inhibited. Not only was there an inhibition of the formation of the multicellular lentoid structures, there was no evidence of even the earliest stages of morphological differentiation such as the formation of the monolayer of epithelium like cells with a cobblestone-type packing (20). These transformed cultures, which appeared similar in morphology to RSV-transformed fibroblasts, continued to replicate and could be carried through at least five passages successfully. It was not possible to obtain a successful passage of normal lens cultures because of the differentiation which had occurred.

Reversibility of the *src* inhibition of lens cell differentiation. Infection of chicken embryo lens cells with a mutant of RSV which is temperature sensitive for pp60$^{v\text{-}src}$, LA24A, demonstrated that the suppression of lens cell differentiation in culture by v-*src* was reversible. The LA24A-infected lens cells exhibited the transformed phenotype at the permissive temperature for transformation, 36°C. These cultures were normally kept through three passages. While maintained at 36°C the cultures showed no sign of morphological differentiation (Fig. 2a). However, they could be shifted to 41°C, the nonpermissive temperature, at any point during the culturing time and result in the initiation of lens cell differentiation. The progression of differentiation of these cells in culture was normal, resulting in the production of lentoids (Fig. 2b). This morphogenic differentiation required 5 to 6 days after the temperature shift, which is similar to the time required for the morphogenic differentiation of freshly isolated lens cells. LA24A-infected cultures which had been allowed to differentiate at the nonpermissive temperature were shifted down to 36°C. The differentiated morphology of the lentoids was lost, and the cultures resembled the original transformed

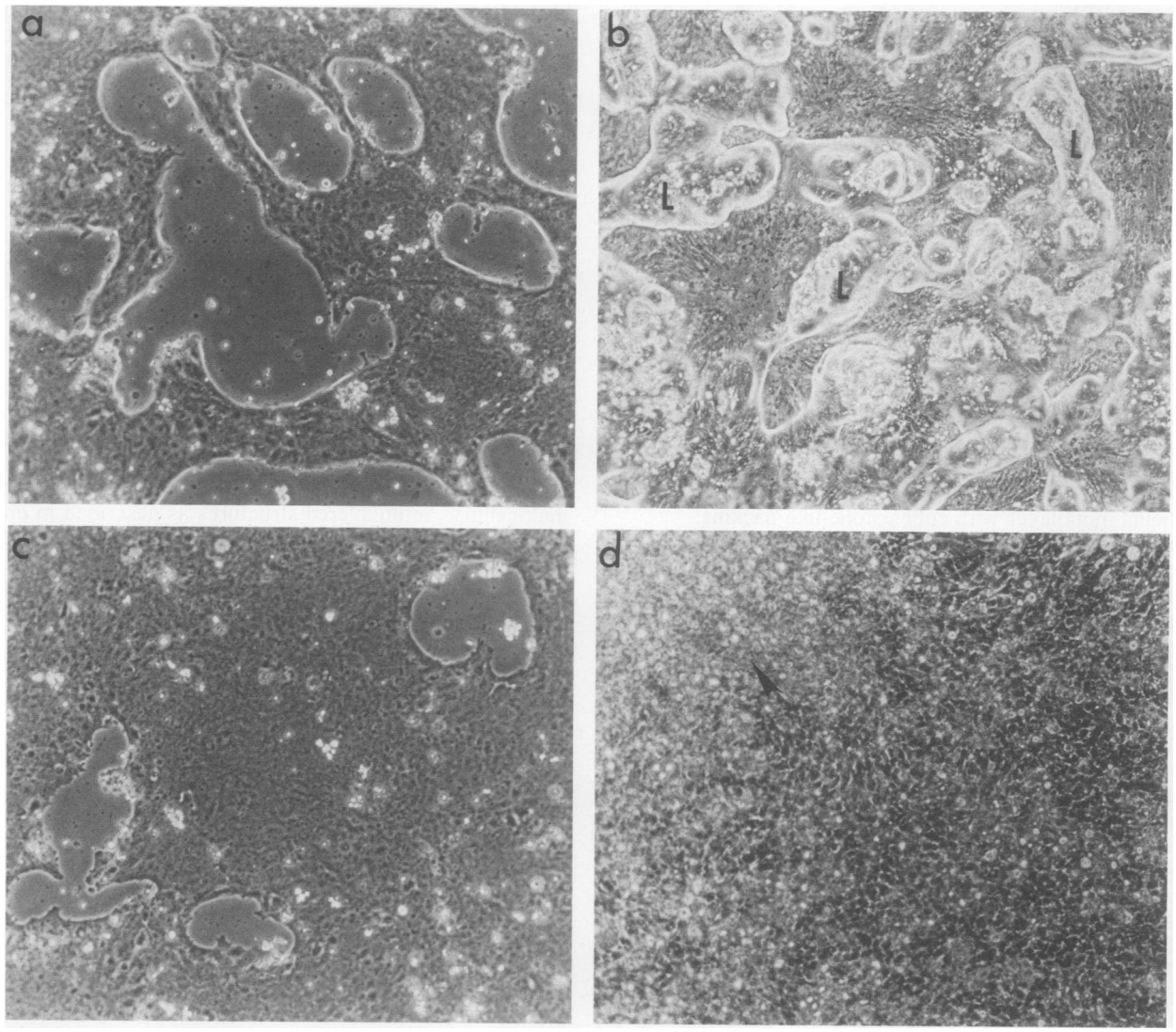

FIG. 1. Infection of lens cells with the v-*src* virus: normal (a, b) and RSV-infected (c, d) chicken embryo lens cultures at different stages of differentiation and infection, respectively. At 2 days after plating the normal (a) and infected (c) cultures are similar. By 5 days after plating the infected cultures (d) are transformed with many areas of piled-up, rounded cells (arrow), whereas the normal cultures (b) have fully differentiated as characterized by the presence of the highly differentiated lentoid (L) structures. Magnification, ×81.

lens cells (Fig. 2c). Thus, the effects of $pp60^{v\text{-}src}$ on lens morphogenesis and differentiation were fully reversible.

Gap junctions in normal and transformed lens cells. Normal lens cultures, when examined at the electron microscopic level, are very similar to normal lens tissue (20, 21). The lentoids which form as the cultures differentiate are comprised of cells which are characterized by an increased cell volume, loss of organelles, and extensive junctional membrane. These characteristics are represented by Fig. 3a, a thin section in which the entire area of plasma membrane apposition seen is junctional and only a part of an even larger junction. Previous reports have extensively characterized the junctions in normal chicken embryo lens cultures (20, 21). RSV-transformed lens cells, when examined as thin sections in the electron microscope (Fig. 3b), were quite unlike the cells found in normal differentiating lens cultures. Most notably, the cytoplasm of the transformed cells remained full of organelles, and the cells demonstrated few intercellular attachments. As well as appearing distinctively different from differentiated lens cells, these cells bore little morphological resemblance to the undifferentiated lens epithelial cells. The cellular morphology and cytoplasmic profile of the transformed lens cells in thin section were similar to those of transformed fibroblasts, except that the cytoskeleton was not disrupted. Budding virus was often detected at the cell surfaces.

In normal lens cultures up to 50% of the plasma membrane is involved in gap junction-like structures (21). These junctions, found in both lens epithelial and fiber cells, are formed by two opposing plasma membranes butting up directly against one another, not from membrane overlapping. This type of junction often results in the long junctions (3 to 5 μm on average [21]) which are characteristic of lens cells in culture as well as in vivo. After transformation of cells with RSV, none of this type of junction could be found. Figure 3b represents a typical apposed membrane in the transformed lens cultures and is characteristic of what was observed during thorough examination of more than 30 sections.

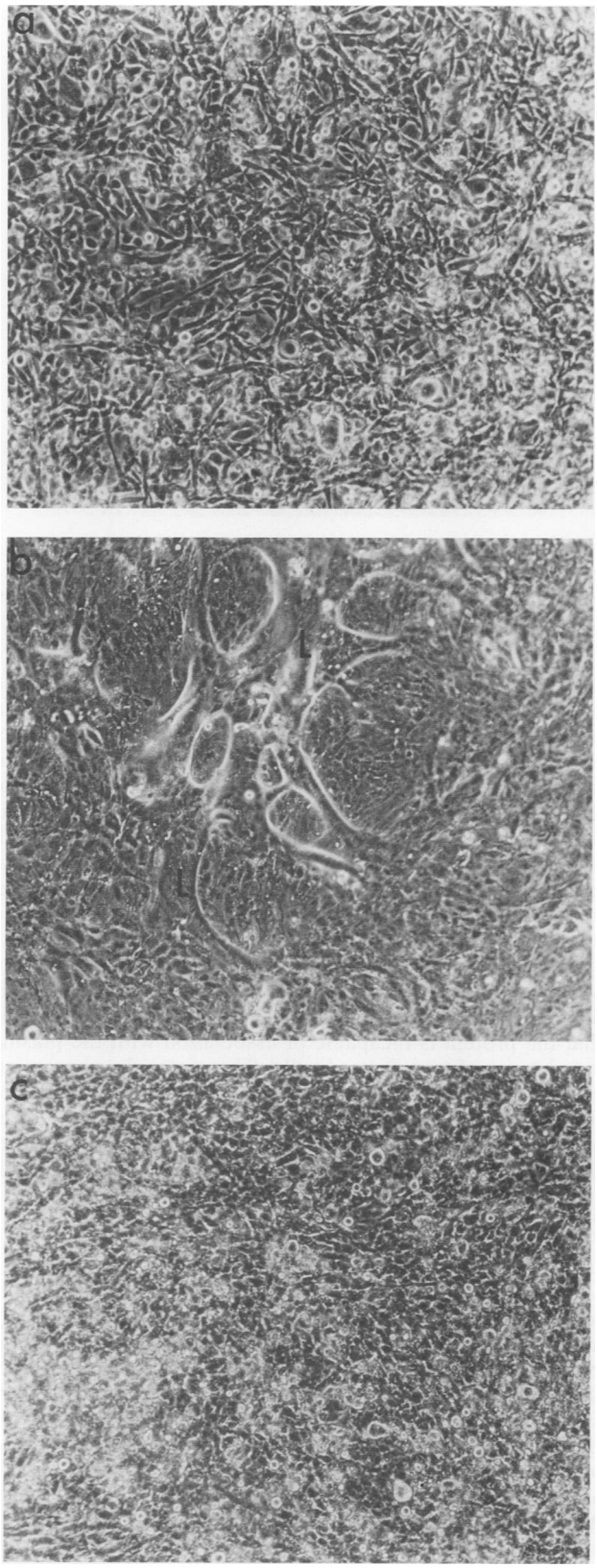

FIG. 2. Transformation of lens cultures by v-*src* is reversible. LA24A-transformed lens cultures (tertiaries) were grown at the (a) permissive (36°C) and (b) nonpermissive (41°C) temperatures for transformation. The cells at the nonpermissive temperature differentiated normally (L denotes lentoid structure). Shifting of cultures which had differentiated at 41°C back down to 36°C (c), under which conditions the v-*src* gene is reexpressed, produced cultures which appeared morphologically identical to those maintained at 36°C. Magnifications, ×81.

TABLE 1. Synthesis of the gap junction protein MP28 in normal and v-*src*-transformed lens cultures

Cells	Temp[a] (°C)	Days in culture[b]	Rate of synthesis of MP28[c]
Lens	36	2	1.0
Lens	36	9	0.2
Lens-24A	41	1	0.6
Lens-24A	41	7	0.4
Lens-24A	36	2	0.6
Lens-24A	36	5	0.8
Lens-24A	41 shifted to 36	2 (after shift)	0.4
Lens-24A	41 shifted to 36	4 (after shift)	0.8

[a] Temperature for transformation (36°C, permissive; 41°C, nonpermissive).
[b] By 5 days normal cultures are differentiated.
[c] All counts are normalized to that of normal lens cultures at 36°C at 2 days in culture, corresponding to early in differentiation.

Although thin sections of the transformed lens cells sectioned both parallel and perpendicular to the substrate were examined, gaplike intercellular junctions were not found. Therefore, RSV transformation of chicken embryo lens cultures results in the inhibition of gap junction formation.

Synthesis of the MP28 protein. MP28 has been identified as the major protein component of the lens gap junctions (9, 27). Since the ultrastructural studies had demonstrated an absence of gap junctions in the transformed cells, it was important to determine whether this was due to a suppression of the synthesis of the lens junction protein or to an inability of the junction protein to assemble properly in the transformed cells.

For these biochemical studies, LA24A-infected lens cultures were grown at both the permissive and nonpermissive temperatures for transformation and were labeled with [^{35}S]methionine for 2 h at different times in culture. Solubilized protein samples were electrophoresed on SDS-polyacrylamide gels, the gels were subjected to autoradiography (Fig. 4), and the individual lanes were scanned by densitometry. The density of the band corresponding to MP28 (determined by comigration with purified MP28) in each lane was quantitated (Table 1). Through the use of Western blot (immunoblot) techniques utilizing the same antibody to MP28 as in the immunofluorescence experiments, it was determined that the band in chicken embryo lens cultures which comigrated with purified MP28 was indeed MP28. In addition, quantitation of MP28 at different stages of differentiation of the lens cultures was similar with either direct protein staining or the Western blot quantitation. In the early stages of differentiation (day 2) MP28 was synthesized at high levels. The rate decreased once the lentoid bodies were formed (by day 9). A parallel decrease in synthesis as differentiation progressed was also observed in infected cultures which were allowed to differentiate at the nonpermissive temperature. In contrast, infected cultures maintained at the permissive temperature continued to synthesize MP28 at levels approaching that of the early lens cultures. Also a downshift of infected, differentiated cultures resulted in an increase in synthesis. Hence the rate of synthesis does

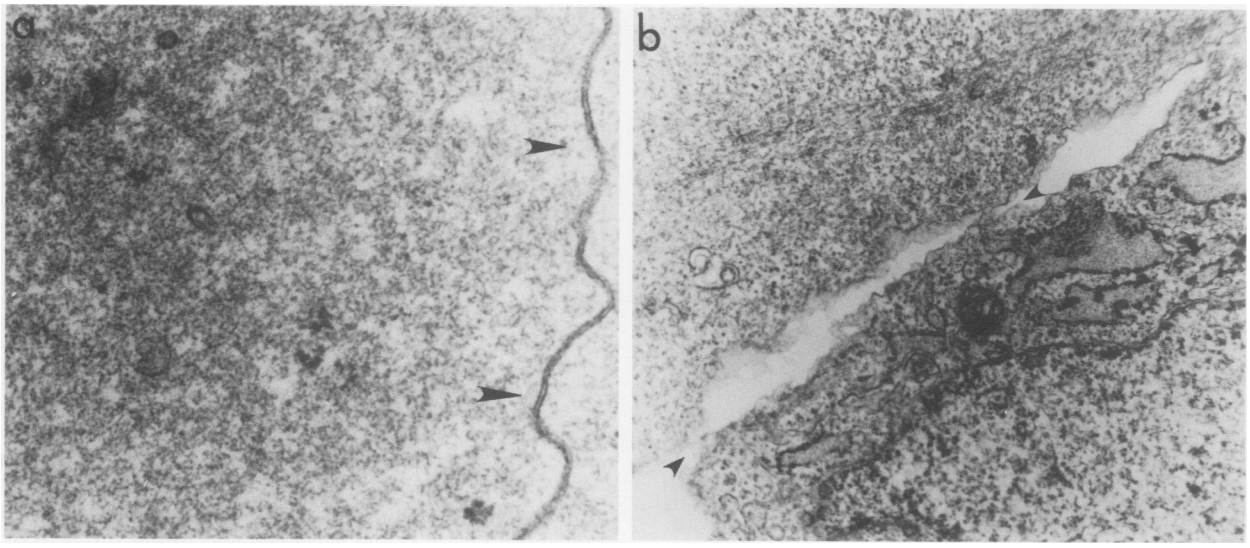

FIG. 3. Junctions in normal and v-*src*-transformed lens cells: an ultrastructural analysis. Normal lens cultures (a), examined as thin sections in the electron microscope, contain extensive regions of junctional membrane (arrows denote junction which spans entire micrograph). No lens gap junctions were found between v-*src*-transformed cells. (b) Typical apposed membrane (arrow) in the transformed lens cultures. Magnification, (a) ×28,500 and (b) ×17,900.

not appear to be affected by the expression of $pp60^{v\text{-}src}$, and the suppression of synthesis of differentiated products which has been demonstrated for the protein products of transformed chondroblast cultures does not apply to MP28. If anything, the synthesis of MP28 is increased in the transformed cultures. Similar studies were carried out for the delta-crystallin protein. As with MP28, synthesis of this protein was maintained at a normal level in the transformed cells (data not shown).

Localization of MP28 in RSV-transformed lens cells. Immunofluorescence was used to determine whether the failure of transformed cells to produce gap junctions resulted from the inability of MP28 to localize to the plasma membrane or to a failure of membrane-associated MP28 to assemble gap junctions. Transformed lens cells were stained with a rabbit antibody to MP28. The staining with this antibody of normal lens cultures was specifically at the plasma membrane as early as the formation of the epithelial monolayer with cobblestone-type packing (20), which is the first morphological alteration know to accompany lens differentiation in culture (Fig. 5a). In contrast, the staining of the transformed cells was light and diffuse with no indication of membrane staining (Fig. 5b). These results show some accumulation of MP28 in the cells, as might be predicted from the synthesis data. The lack of membrane localization of MP28 indicates that the protein is incapable of association with the plasma membranes of transformed cells. Therefore, the expression of $pp60^{v\text{-}src}$ either alters the distribution of this protein and thus prevents the assembly of the gap junction structure, alters the protein so that it cannot assemble into the junctional structures, or makes the gap junction structures unstable so that they can not accumulate.

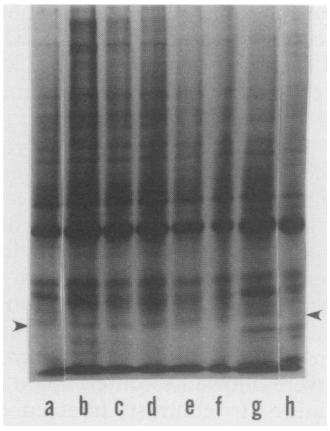

FIG. 4. Polyacrylamide gel analysis of MP28, the major lens gap junction protein, in normal and v-*src*-transformed lens cultures. [^{35}S]methionine-labeled proteins were electrophoresed on a Laemmli 10% polyacrylamide gel and subjected to autoradiographic analysis. Lanes: LA24A-infected lens cultures after 1 (a) and 7 (b) days at the nonpermissive temperature (41°C) for transformation; infected lens cultures first grown at 41°C, to allow differentiation to occur, and then switched to the permissive temperature for v-*src* for 2 (c) and 4 (d) days; infected lens cultures after 2 (e) and 5 (f) days at the permissive temperature for transformation (36°C); control, uninfected lens cells grown for 2 (g) and 9 (h) days in primary culture. Arrows denote position of MP28.

DISCUSSION

Chicken lens epithelial cells can be infected and transformed by RSV. The transformed cells are morphologically similar to other cell types, including fibroblasts, transformed by RSV. The transformation also disrupts the expression of the normal differentiation program of the cells. This suppression of the expression of normal cell differentiation parallels the results which have been reported for other cell systems, including retinal melanoblasts (7, 26), myoblasts (11, 14, 22), and chondroblasts (1, 3, 23). In the myogenic and chondrogenic cells the suppression of differentiation is seen as a suppression of the synthesis of normal differentiation products. In contrast, the synthesis of the major junctional protein MP28 was not reduced by transformation. In spite of the continued synthesis of MP28 the transformed cells only exhibited a light staining for MP28 in comparison to the control cells, and there was no localization of the MP28 at

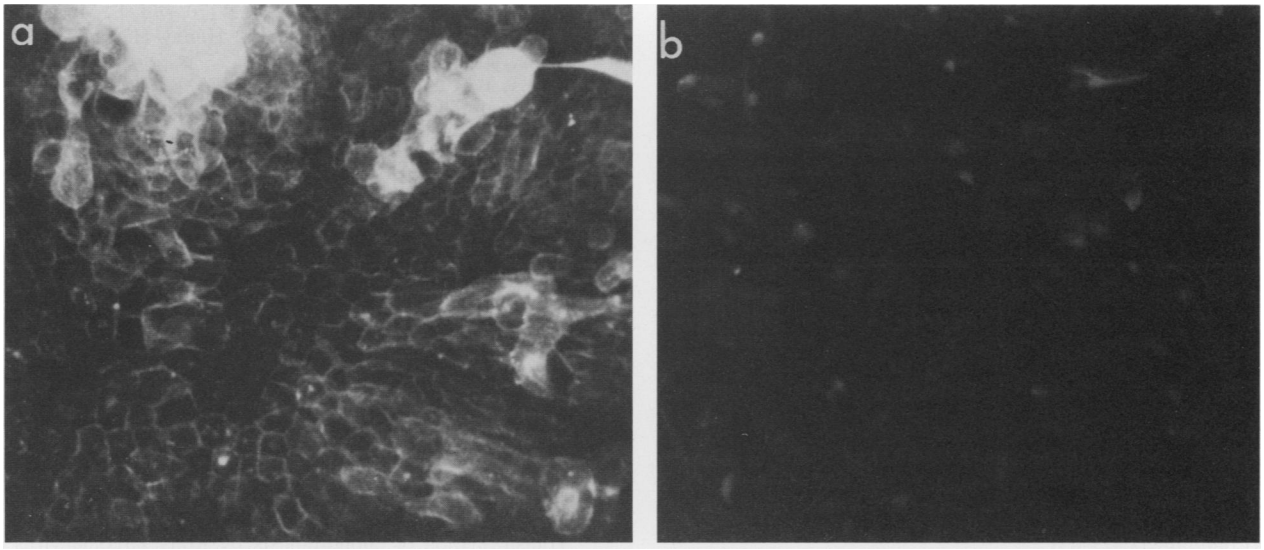

FIG. 5. Immunofluorescence localization of MP28 in v-*src* transformed cells. In normal lens cultures, even at the earliest stage of differentiation (a), MP28 is found localized to the plasma membrane. Staining for MP28 in v-*src*-transformed lens cultures (b) was light and diffuse with no indication of membrane staining. Magnification, ×780.

the plasma membrane. This suggests that a primary defect in the differentiation of the RSV-transformed lens cells was a failure to partition the MP28 to the plasma membrane and assemble it to form gap junctions. The absence of the gap junctions was also confirmed by electron microscopic examination. It is likely that the formation of the normal multilayered lens structures in vivo or the lentoid bodies in vitro depends on the formation of the lens junctions to provide both the proper structural relationships and the essential cell-to-cell communication afforded by the gap junctions. Hence it is not surprising that lens morphogenesis is inhibited when junction formation is prevented.

There are several mechanisms which may account for the ability of $pp60^{v\text{-}src}$ to block junction formation. The first candidate would be the phosphorylation of MP28. It has been reported that MP26, the protein analogous to MP28 in bovine lens, is a phosphoprotein, but the phosphorylation detected was primarily on serine and not on tyrosine, the substrate for the v-*src* kinase (15). This study used differentiated lens tissue in which the junctional complexes must already be assembled in the membrane. The sequence data on MP26 show a number of tyrosine residues which reside primarily in or at the ends of the proposed transmembrane domains (13). It is possible that these residues may be phosphorylated by $pp60^{v\text{-}src}$ as the protein is made and that such phosphorylation prevents its assembly into gap junctions. Alternatively, $pp60^{v\text{-}src}$ could alter proteins in the membrane, including other junctional proteins, which results in blocking the assembly of the junctions. It is possible that the phosphorylation of tyrosine residues plays an important role in the normal regulation of the gap junction channels; previous data with v-*src*-dependent phosphorylation indicate that such phosphorylation closes these channels (4). Therefore, the inability to find phosphorylation of tyrosine in the bovine lens study (15) may simply result from the fact that the junctions in mature lens tissue are held in the open position and the tyrosine sites are already phosphorylated.

Recent studies from this laboratory have demonstrated that the extracellular matrix receptor integrin is a control point for myogenic differentiation (19). Since this protein is a target for v-*src* it is possible that the effects reported here are also mediated by the phosphorylation of integrin. This step may reduce the affinity of integrin for the extracellular matrix components. We hypothesize that the anchoring of the cells to the proper substrate (in this case, probably laminin) is essential for the stabilization of the complex morphology of the lens. Once these connections are severed, the internal components such as the junctions may no longer find the proper nucleation points required for assembly, or the junctional assemblies which are formed may not be stable. Inhibition of junction formation in the lens would deny the cells a means of both communication and nutrient transfer which are essential to maintain the differentiated lens cells. The failure to form the junctions could also provide a means of blocking further differentiation by denying the cells the signals normally transmitted via the gap junctions.

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