Nonmitogenic Morphoregulatory Action of pp60^{v-src} on Multicellular Epithelial Structures

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Madin-Darby canine kidney (MDCK) cells form polarized, multicellular epithelial structures in vitro. Low-level expression of pp60^{v-src} in MDCK cells elicits plasticity in these multicellular structures. Plasticity was revealed by the displacement of cells from mechanically stressed regions of the epithelial monolayers; however, the two-dimensional relationship between the cells in the remainder of the monolayer was maintained. Electron microscopy of multicellular structures revealed abnormal separation of the lateral membranes of adjacent cells and selective uncoupling of the junctional complex; the zonula adherens was disrupted, but the zonula occludens and desmosomes were retained. Significantly, this result was not accompanied by transformation of the cells, as judged by the absence of anchorage-independent growth potential. These results demonstrate a nonmitogenic biological activity of pp60^{v-src} which is experimentally dissociable from transformation. This morphoregulatory action on higher-order epithelial structures may reflect a function of related cellular tyrosine kinases.

The biological action of the viral tyrosine kinase, pp60^{v-src}, has been linked invariably to uncontrolled cell proliferation (5; for reviews, see references 3 and 31). However, the cellular homolog, pp60^{c-src}, is expressed most abundantly in nonproliferating tissues. The highest levels of pp60^{c-src} in vertebrate tissues have been demonstrated in postmitotic, terminally differentiating neurons (7, 13, 18, 44, 47, 64). A similar pattern of pp60^{c-src} and *c-src* RNA transcripts has been demonstrated in invertebrate species (60, 62). In addition, pp60^{c-src} or *c-src* RNA has been detected in nearly all tissues examined, including muscle, nonneural epithelia, and connective tissue (13, 22, 34, 44, 60, 62).

The fact that pp60^{c-src} expression is particularly high in postmitotic cells implies that pp60^{c-src} does not stimulate cell proliferation in these tissues, an implication which has been difficult to reconcile with the known action of pp60^{v-src}. This paradox has led to the question of whether pp60^{c-src} and pp60^{v-src} have qualitatively similar actions. This question has been addressed almost exclusively from the perspective of transformation by comparing pp60^{c-src} or pp60^{v-src} variants with pp60^{v-src} for the potential to cause uncontrolled proliferation of cultured fibroblasts, as assayed by anchorage-independent growth, focus formation, or tumorigenesis (32, 44, 55, 61). These studies have shown that elevated levels of pp60^{c-src} cannot fully transform cultured fibroblasts, although extremely high levels have been shown to have weak focus-inducing activity in rodent fibroblasts (36). Thus, pp60^{c-src} can partially mimic the transforming action of pp60^{v-src}, but only under exceptional circumstances.

Conversely, since the *v-src* gene apparently arose from the *c-src* gene (66, 67), one could ask whether pp60^{v-src} has retained the potential to mimic biological actions that might normally be performed by pp60^{c-src}. At present, this cannot be tested directly because the function of pp60^{c-src} is unknown. However, the developmental pattern of pp60^{c-src} expression in a wide variety of tissues suggests that nonproliferative actions of pp60^{c-src} or pp60^{v-src} would manifest themselves more overtly in differentiated tissues, which have established a highly organized histoarchitecture, than

in cultured fibroblasts. These theoretical considerations provide a rationale for assaying pp60^{v-src} for activities other than transformation or increased cell proliferation in highly differentiated cells which are capable of forming tissue-like structures.

For this purpose, Madin-Darby canine kidney (MDCK) cells have been used because they retain the morphogenetic determinants required to form polarized multicellular epithelial structures in vitro. These structures exhibit many biochemical, functional, and architectural properties of their tissue of origin, renal tubular epithelium (8, 9, 50, 58, 69). The cells form a continuous monolayer which assumes a planar conformation in petri dishes and form closed cystic structures in collagen gels (25). Within these structures, the cells exhibit a polarized distribution of enzymes between the apical and basolateral membranes and the ability to vectorially transport ions and solutes (8, 9, 50, 69).

The results of the study reported here show that low-level expression of pp60^{v-src} in MDCK cells elicits plasticity in multicellular, polarized epithelial structures in vitro; mechanically stressed regions of those structures are deformed, but other regions show no apparent disruption of the multicellular, polarized organization normally exhibited by the cells. This plasticity coincides with decreased cell-cell contact and the selective disruption of one component of the junctional complex, the zonula adherens. Significantly, this phenotype is expressed without transformation of the cells, as judged by the absence of anchorage-independent growth potential. These results demonstrate that pp60^{v-src} has a nonproliferative morphoregulatory action in higher-order epithelial structures and suggest that pp60^{v-src} has retained the ability to act on morphogenetic determinants that may be controlled normally by a related cellular tyrosine kinase(s).

MATERIALS AND METHODS

Vector constructions. The T4 DNA ligase, Klenow fragment of *Escherichia coli* DNA polymerase I, *Bam*HI linker, and all restriction enzymes were purchased from New England Biolabs. The bacterial alkaline phosphatase was from International Biotechnologies Inc.

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Details of pFVXMneo and pMvsrc have been described elsewhere (36, 40). Vectors were constructed by techniques described previously (48). Briefly, the v-src coding sequence for all vectors was derived from pMvsrc; the *Nco*I site within the ATG start codon was cut, blunt-ended by the Klenow reaction, and ligated to a dodecameric *Bam*HI linker; *Bam*HI and *Bgl*II digestion yielded a 1.7-kilobase (kb) fragment which contained the intact v-src coding sequence. Plasmids pMOvsrcSVneo(+) and (-) were each made in two steps. First, the *Bam*HI-*Bgl*II v-src restriction fragment was ligated into the *Bgl*II site of pFVXM to yield pMOvsrc (data not published). Second, a 2.3-kb *Bam*HI fragment from pSVneo, which contains the transposon Tn5 Neo^r coding sequence under the control of the simian virus 40 (SV40) early-region enhancer promoter segment, was ligated into the *Bgl*II site of pMOvsrc in both orientations. The pMOneoMT₂₁₁ v-src plasmid was constructed by the ligation of two fragments: a 7.7-kb *Bam*HI-*Xho*I segment from pRNSV0X, which provided the long terminal repeats (LTRs), ψ site, transposon Tn5 neo^r gene, the plasmid replication origin, and Amp^r gene (pRNSV0X was provided by M. Kreigler and was derived from pFVXM [40]) and a 1.9-kb *Bam*HI-*Sal*I insert from pUCMT₂₁₁ v-src (data not shown), which provided the truncated metallothionein I promoter and the v-src coding sequence.

Generation of virus stocks. Virus stocks were prepared from ψ -2 cells (49). Cells which had been transfected with plasmid DNA with polybrene (38) were selected for 1 week in the toxic aminoglycoside G418 (400 μ g/ml; Geneticin; Sigma Chemical Co.) and pooled. ψ -2 cells can package defective Moloney murine retrovirus genomic transcripts into infectious ecotropic virus particles. Ecotropic virus was harvested and used to infect an amphotropic packaging cell line, ψ AM (11). These cells were selected in G418 (400 μ g/ml) for 1 week and pooled. The infectious, amphotropic virus stock produced by these cells was used to infect the MDCK cells, which were selected subsequently in G418 (400 μ g/ml) for 1 week. The pMvsrc plasmid was transfected into ψ AM cells, which resulted in multiple transformed foci; these were enriched by selective trypsinization and pooled as a source of amphotropic Mvsrc virus.

Southern analysis. High-molecular-weight DNA was isolated from MDCK cells and cut with *Eco*RI. The restricted DNAs (15 μ g) were applied to a 0.8% (wt/vol) agarose gel, electrophoresed, and transferred to nitrocellulose paper as described (48). Filters were hybridized at 42°C for 24 h to $\sim 10^8$ cpm of ³²P-labeled, nick-translated pMOvsrcSVneo(-) DNA (1 μ g). Filters were washed extensively in 0.1 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-1% sodium dodecyl sulfate (SDS) at 55°C and exposed to Kodak XAR-5 film, with an intensifying screen, for 24 h at -70°C.

Cell culture. The MDCK cells used in this study were cloned from an original low-passage-number stock and have been characterized previously (52). Cells were grown in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere containing 5% CO₂.

Polyp formation and the shedding of cells from confluent MDCK cell monolayers were detected as follows. Cells (5×10^5) were plated in a 60-mm plastic petri dish (Nunc) and allowed to grow to confluency. The medium was replaced with complete medium (DMEM with 10% FBS) containing 0.33% agar (Difco) (day 1). The cells were refed with 3 ml of agar-containing complete medium on day 3 and photographed on day 7. In some cases, polyps were isolated from

the soft agar by dilution with complete medium (DMEM-FBS) and replating on plastic petri dishes.

Multicellular cysts were grown in a collagen matrix as described (74). Briefly, 3×10^5 single cells of each clone were mixed with 1 ml of uncongealed collagen gel matrix (~ 0.7 mg of collagen per ml in DMEM-FBS) and cast into a 35-mm petri dish (Nunc). Cultures were fed with fresh medium every 3 to 4 days and photographed on day 14. Collagen was prepared aseptically from rat tail tendons as described (51).

Disk-shaped multicellular cysts were made essentially as described (25) but with modifications. Collagen gel matrix (3 ml with DMEM, 10% FBS, and antibiotics) was allowed to congeal in a 60-mm petri dish (Nunc); 2.5×10^4 cells were plated onto this surface and allowed to grow for 3 days into flat colonies of 5 to 10 cells, and then 2 ml of additional collagen gel matrix was cast on top of these colonies. Colonies formed disk-shaped cysts during the next 2 days (25). The cysts were photographed and processed for light microscopy of 3- μ m sections 5 days after the top layer of collagen was added.

pp60^{v-src} kinase assay. MDCK cells were subcultured at the same density (8×10^4) on 35-mm plastic petri dishes in triplicate and allowed to grow for 3 days as described above. Cells were washed in ice-cold 15 mM Tris hydrochloride (pH 7.5)-120 mM NaCl and lysed in 1% Nonidet P-40-1% sodium deoxycholate-0.1% SDS-0.15 M NaCl-10 mM sodium phosphate buffer (pH 7.2)-2 mM EDTA (RIPA buffer [21]). Following centrifugation at $12,000 \times g$ for 5 min, the cell extracts were normalized to total protein (BioRad protein assay with bovine serum albumin as the standard). pp60^{v-src} was immunoprecipitated from the cell extracts essentially by the method of Brugge and Erikson (5), except that protein A-Sepharose 4B beads (Pharmacia) were substituted for Formalin-fixed protein A-bearing *Staphylococcus aureus*, and a mouse monoclonal anti-pp60^{v-src} antibody (Oncor) was used instead of tumor-bearing rabbit (TBR) serum (provided by J. S. Brugge [6]). The kinase assay was performed on the resulting immunoprecipitates with [γ -³²P]ATP (4,000 Ci/mmol; New England Nuclear) as described by Collett and Erikson (10); the beads were washed twice in HS-B buffer (52) containing 2 mM ATP. The immunoprecipitates were boiled in SDS sample buffer and applied to 7.5 to 15% linear gradient SDS-polyacrylamide gels (41) as described and modified (52); gels were fixed and stained with Coomassie brilliant blue. The destained gels were dried and exposed to XAR-5 X-ray film for 2.5 h at -80°C with an intensifying screen. For quantitation, the band corresponding to the immunoglobulin G (IgG) heavy chain was excised, placed in 10 ml of Aquasol, and counted in a scintillation spectrometer.

Metabolic labeling. Subconfluent cultures of each clone were grown for 3 days on 35-mm petri dishes in DMEM-FBS from an initial cell density of 8×10^4 cells. For metabolic labeling, the medium was discarded and the cells were preincubated in 2 ml of MEM-Met (minimum essential medium without methionine and supplemented with 2.5% dialyzed FBS) for 15 min at 37°C. The medium was discarded, and the cells were labeled in 1 ml of MEM-Met with 160 μ Ci of [³⁵S]methionine ($\sim 1,200$ Ci/mmol; New England Nuclear) for 10 h at 37°C. The medium was discarded, and the cells were washed twice with ice-cold Tris-saline-phenylmethylsulfonyl fluoride and extracted with 400 μ l of RIPA buffer (21) for 15 min at 4°C. The extract was centrifuged at $48,000 \times g$ for 10 min. The extracts were normalized to total trichloroacetic acid (TCA)-precipitated counts, and 5

μ l of anti-pp60^{v-src} monoclonal antibody (McAb) 327 (46), 5 μ l of McAb used for the kinase assay, or 3 μ l of TBR serum was added. Following incubation for 40 min at 4°C, 30 μ l of a 10% (wt/vol) suspension of Formalin-fixed protein A-bearing *S. aureus* was added; for immunoprecipitation of the McAbs, the bacteria were preincubated with 1 μ g of rabbit anti-mouse IgG per reaction volume. After 20 min at 4°C, the bacteria were collected by centrifugation at $12,000 \times g$ for 3 min and washed by vortexing in three changes of RIPA buffer (21) and two changes of a buffer containing 1 M NaCl, 10 mM Tris hydrochloride, pH 7.5, and 0.5% (wt/vol) Triton X-100. The immunoprecipitates were processed subsequently for SDS-polyacrylamide gel electrophoresis (PAGE) and fluorography as described previously (52). The X-ray film was preflashed to an absorbance of ~ 0.15 at 514 nm (42), and the level of [³⁵S]methionine incorporation was determined by analyzing the resulting fluorogram in a Beckman DUV-7 spectrophotometer equipped with a scanning densitometer.

Light microscopy of Plexiglas-embedded sections. Multicellular cysts (grown in collagen matrix) were fixed in 1% glutaraldehyde (Electron Microscopy Sciences) in phosphate-buffered saline (PBS) and embedded in Plexiglas (Rohm and Haas) as described (23). Sections (3 μ m) were cut with an MT2B ultramicrotome (Sorvall) and applied to polylysine-coated cover slips. These sections were stained with toluidine blue and photographed with an Ultrastar light microscope (American Optical).

Electron microscopy. MDCK cells were fixed in a solution containing 1.25% glutaraldehyde and 1% OsO₄ in 50 mM PIPES [piperazine-*N,N*-bis-(2-ethanesulfonic acid), pH 7.2] for 5 min at room temperature. The cells were washed twice in 50 mM PIPES (pH 7.2) for 5 min each and dehydrated sequentially for 5 min: once in 60% ethanol; once in 2% uranyl acetate in 60% ethanol; once in 80% ethanol; twice in 95% ethanol; five times in 100% ethanol; and twice in 100% propylene oxide. The cells were embedded in Epon-Araldite. Silver sections were cut with an LKB ultramicrotome, stained in uranyl acetate-lead citrate, and viewed in a Philips 420 transmission electron microscope operated at 80 kV.

Indirect immunofluorescence. Cells were grown on collagen-coated cover slips. The cells were fixed with 1.75% (wt/vol) formaldehyde in PBS, washed in PBS for 10 min, and then permeabilized with PBS containing 0.5% (vol/vol) Triton X-100 and 2 mM MgCl₂ for 2 to 15 min at room temperature. Cells were incubated in a 1:10 dilution of nonimmune serum or McAb anti-pp60^{v-src} IgG (Oncor) in PBS for 30 min at 37°C in a humidified atmosphere. The cells were washed extensively with PBS and then incubated in a 1:150 dilution of affinity-purified goat anti-mouse IgG conjugated with rhodamine (Boehringer Mannheim) in PBS for 30 min at 37°C in a humidified atmosphere. Following extensive washing in PBS, cover slips were mounted in Elvanol, viewed with a 63 $\times$ objective in a Zeiss Universal microscope equipped with epifluorescence illumination, and photographed on Tri-X Pan film.

RESULTS AND DISCUSSION

Polarized epithelial cells expressing pp60^{v-src}. The first experiments were performed to determine whether *v-src* could alter the growth properties of MDCK cells. Subsequently, we sought to reduce pp60^{v-src} expression to a level below that which leads to transformation of MDCK cells. This strategy is related conceptually to experiments which have

FIG. 1. (A) Retrovirus vector constructions. Schematic map of the provirus insert from each plasmid is shown. The plasmid designations are given at the left. The names of the MDCK cell lines which were derived from infection by the corresponding viruses are given in parentheses. pMvsrc contains only the Schmidt-Ruppin-A *v-src* coding sequences. pMOvsrcSVneo(+) and pMOvsrcSVneo(-) each contain an additional transcriptional unit composed of the SV40 early region enhancer-promoter segment; the Tn5 aminoglycoside phosphotransferase (Tn5 neo^r) coding region; and the SV40 small t intron and polyadenylation sequences. This SVneo unit is in either the same (+) or opposite (-) transcriptional orientation as the upstream LTR promoter. The pMOneoMT₂₁₁ *v-src* provirus has the Tn5 neo^r coding sequence under the control of the 5' LTR promoter. The *v-src* coding sequence is placed immediately downstream from a segment which contains 211 base pairs of the mouse metallothionein I promoter. The pFVXMneo provirus encodes only the Tn5 neo^r gene. Symbols: open boxes, LTR sequences; open circle, virus genome packaging signal, ψ ; open arrowhead, 5' splice site; solid arrowhead, 3' splice site; solid rectangles with solid circles, SV40 early region enhancer-promoter segment; stippled box, partial sequences of the mouse metallothionein I promoter; open circle with tail, SV40 small t polyadenylation sequence; thick lines, rat genomic DNA derived from original provirus clone; E, *Eco*RI restriction site. (B) Southern blot analysis of pp60^{v-src}-expressing and control MDCK cell lines. High-molecular-weight DNA was digested with *Eco*RI, electrophoretically separated, transferred to nitrocellulose, and hybridized with a ³²P-labeled nick-translated pMOvsrcSVneo(-) plasmid. Lanes: m, λ HindIII relative molecular size markers (a, 23 kb; b, 9.4 kb; c, 6.6 kb; d, 4.4 kb; e, 2.3 kb; f, 2.0 kb); 1, MFneoPOP; 2, M(-)VB; 3, MMTv-10 (partial digestion); 4, M(+)V13; 5, MMTv-7; 6, Mvsrc-9.

demonstrated that a narrow range of pp60^{v-src} levels defines a threshold for transformation of rodent fibroblasts (35).

The Schmidt-Ruppin A strain *v-src* gene was stably introduced into MDCK cells by using helper-free, defective, amphotropic retroviruses. Initially, MDCK cells were infected with a simple virus in which transcription of the *v-src*

TABLE 1. Characterization of cell lines

Cell line	Relative $pp60$ kinase level	Anchorage-independent growth (no. of colonies/dish) ^a	Doubling time (h)	Cell shedding	Polyp formation	Spike formation in collagen gels	Disruption of cell-cell contacts ^b
MFneoPOP (control)	1	4 ^c (<0.001%)	28	—	—	—	—
M(-)VB	2.5	0 ^c (0%)	25	+	—	—	+
M(+)V13	9	1 ^c (<0.001%)	34	+	—	+	+
MMTv-10	11	2 ^c (<0.001%)	27	+	—	+	+
MMTv-7	16	2 ^c (<0.001%)	26	+	—	+	+
Mvsrc-9	50	420 (2.8%)	ND ^d	+	+	NA ^e	+

^a Cloning efficiencies are given in parentheses.

^b Includes lateral membrane separation and disruption of the zonula adherens as determined by electron microscopy (see Fig. 6).

^c These colonies were cystic and arose from cell aggregation at the time of plating; unlike the solid colonies of Mvsrc-9, these cystic colonies exhibited negligible growth during the assay period.

^d ND, Not determined.

^e NA, Not applicable; Mvsrc-9 cells form disorganized, noncystic masses in collagen matrix.

gene is controlled by the Moloney LTR promoter (Mv-src, Fig. 1A). Subsequently, infected cells were selected for growth in soft agar, a condition in which MDCK cells do not normally grow (Table 1). Two weeks later, multiple macroscopic colonies appeared, of which nine were isolated and expanded for analysis. The cells resembled fibroblasts when plated at low densities (<10⁴/cm²; Fig. 2f) and did not form the tight colonies which are a distinctive feature of normal MDCK cell growth (Fig. 2a); but as cell-cell contacts were established, due to increased cell density, the cells appeared to form an epithelial monolayer (Fig. 2g, left). After five to seven passages, every clone that had been selected for growth in soft agar gave rise to highly transformed variant populations of small, round, highly refractile cells which exhibited little cell-cell contact (Fig. 2g, right). These results show that high levels of v-src expression can lead to transformation of MDCK cells (see below). The next step was to construct new vectors designed to reduce the level of $pp60^{v-src}$ expression and hence avoid transformation of MDCK cells.

These vectors enabled selection of infected cells for resistance to the aminoglycoside G418 rather than for augmented growth properties (Fig. 1A). Moreover, they were made with three features designed to reduce the level of $pp60^{v-src}$ expression (see Materials and Methods). First, each construct contains a synthetic linker that alters a consensus leader sequence preceding the ATG start codon, which should theoretically reduce the translation efficiency of the predicted mRNA (39). Second, in pMOneoMT₂₁₁ v-src, the v-src gene is positioned downstream of a truncated mouse metallothionein I internal promoter, which was chosen due to its low constitutive level of expression (4; data not shown). Finally, these are bicistronic retroviral vectors; when selected for the expression of one gene (e.g., Tn5 neo^r), expression of the second gene (e.g., v-src) is commonly suppressed (15). Although we have not analyzed the effect of these features on transcription of the v-src gene, our results show that in all cases there was a substantial reduction in the levels of $pp60^{v-src}$ expressed compared with pMv-src, as required for the present study (see below).

MDCK cells were infected with each of the new viral stocks [MOvsrcSVneo(+), MOvsrcSVneo(-), and MOneoMT₂₁₁v-src] and selected in G418. Cell lines generated by infection with these viruses have been stable for >30 passages without the appearance of transformed variants. The G418^r colonies exhibited different morphological phenotypes, and ~40 clones were isolated (data not shown); of these, 4 clones were subjected to detailed analyses as representing the spectrum of these phenotypes (Fig. 2). In addition, MDCK cells were infected with an FVXMneo

FIG. 2. Morphology and growth properties of $pp60^{v-src}$ and control MDCK cell clones. Cells were subcultured in plastic petri dishes at similar initial densities (~10⁴/cm²) and photographed 3 days later with phase contrast optics (a to g). Cells were allowed to grow to confluency and then overlaid with 0.33% agar and photographed 7 days later (h to j). a, MFneoPOP; b, M(-)VB; c, MMTv-10; d, M(+)V13; e, MMTv-7; f, Mvsrc-1; g, highly transformed variant cell population (right) within a monolayer of Mvsrc-4 cells (left); h, MFneoPOP; i, M(-)VB; j, Mvsrc-9. Arrows in panel i show groups of degenerating single cells which have been shed from the monolayer. Arrows in j show a polyp which has overgrown the monolayer of Mvsrc-9 cells. Bars, 100 μ m.

virus (Fig. 1A), which encodes the Tn5 neo^r (G418^r) gene; cells were selected in G418 and pooled as a control (MFneoPOP cells). These cells exhibited growth properties and a morphology which were indistinguishable from those of the parent clone of MDCK cells (Fig. 2a) (52).

At low cell density, M(-)VB cells formed tight colonies which resembled those formed by the MFneoPOP cells (compare Fig. 2a and b). MMTv-7 cells also formed discrete colonies when plated at low cell densities; however, the cells were more spread out and showed bright, refractile intercellular spaces (Fig. 2e). The M(+)13 cells and MMTv-10 cells exhibited intermediate phenotypes (Fig. 2c and d). All of these cell lines formed a continuous monolayer at moderate cell densities ($\sim 2 \times 10^5/\text{cm}^2$) which closely resembled that formed by the parent MDCK cells (data not shown; see below).

The presence of a proviral insertion(s) in the genome of these clones was demonstrated by Southern analysis. High-molecular-weight DNA from each clone was digested with *Eco*RI and hybridized to a nick-translated plasmid, pMOvsrcSVneo(-) (Fig. 1B); each provirus sequence contained a single *Eco*RI restriction site (Fig. 1A). Clone MMTv-7 had four unique bands, indicating two inserted proviruses, whereas all other clones had two unique bands, indicating single insertions (MMTv-10 showed partial digestion; Fig. 1B, lane 3). Hybridization with separate nick-translated *v-src* and SVneo probes supported this conclusion (data not shown). A faint band with an apparent molecular size of ~ 3 kb was present in all clones, including the control, and presumably represents the canine *c-src* gene.

The presence of a *src* gene product in these clones was determined by in vitro kinase activity and metabolic labeling (Fig. 3). For the kinase assay, extracts from clones grown to approximately the same subconfluent cell densities were normalized to total protein and immunoprecipitated with a McAb raised against pp60^{v-src}. In vitro phosphorylation of IgG heavy chains was determined as described previously (5). All clones exhibited levels of kinase activity greater than that of the MFneoPOP control cells (Fig. 3A). Kinase activity was consistently found in immunoprecipitates of MFneoPOP cell extracts, but longer exposures of the autoradiograms than that shown here (2.5 h) were needed (data not shown). For quantitation of the relative levels of pp60^{v-src} kinase activity, the total pp60 kinase level of each clone was divided by the level of pp60^{v-src} kinase activity of the MFneoPOP control cells (Table 1). A wide spectrum of relative kinase activities was expressed by these clones. Mvsrc-9 cells expressed the highest relative level. The G418^r clones expressed much lower relative levels than Mvsrc-9; the M(-)VB level was only 2.5 times higher than that of MFneoPOP (Fig. 3A and Table 1). No kinase activity was detected in immunoprecipitates of these clones with nonimmune sera (data not shown). The differences in the relative levels of in vitro kinase activity between the clones did not appear to be affected by the growth state of the cells, since a similar spectrum of activities relative to that of MFneoPOP control cells was detected in immunoprecipitates of subconfluent cultures and continuous monolayers of cells (data not shown).

A *src* gene product was also detected in these clones by metabolic labeling and immunoprecipitation. In these experiments a TBR anti-pp60^{v-src} serum and two McAbs raised against pp60^{v-src} (McAb 327 and the McAb used for the kinase assay) were used; similar results were obtained with each antibody preparation, and the results of the McAb 327 immunoprecipitations are presented (Fig. 3B). Clones grown

to the same subconfluent cell densities were labeled metabolically for 10 h with [³⁵S]methionine, and cell extracts normalized to total TCA-precipitated counts were immunoprecipitated. The resulting fluorogram (Fig. 3B) shows that the levels of [³⁵S]methionine incorporation into pp60^{v-src} varied among the clones. All clones derived by infection with viruses encoding *v-src* had levels higher than that in MFneoPOP (Fig. 3). The highest level was found in Mvsrc-9, and lower levels were found in the G418^r clones. These differences did not appear to be the result of a variation in the metabolic activity of each clone, since the total level of [³⁵S]methionine incorporation by the clones varied by <15% (data not shown). In general, the spectrum of pp60^{v-src} expression determined by metabolic labeling reflected that found by the kinase assay. An exception was MMTv-7, which had a higher level of kinase activity than M(+)V13 and MMTv-10, but a lower level of [³⁵S]methionine incorporation. The reason for this difference is at present unknown and is currently under investigation.

Together these results indicate strongly that increased levels of pp60 kinase activity in clones derived by infection with viruses encoding *v-src* were the result of the introduced *v-src* gene and subsequent expression of active pp60^{v-src}. In particular, the high ratio of the level of kinase activity to the level of synthesis in *v-src*-infected clones compared with

FIG. 3. (A) pp60 kinase assay of pp60^{v-src}-expressing and control MDCK cell lines. The protein kinase assay with [γ -³²P]ATP was performed on pp60^{v-src}-specific immunoprecipitates prepared from each MDCK cell line normalized to total protein. The immunoprecipitates were applied to 7 to 15% linear gradient SDS-polyacrylamide gels and electrophoresed. The resulting autoradiograms reveal specific ³²P labeling of the IgG heavy chains (arrow). Lanes: 1, MFneoPOP; 2, M(-)VB; 3, MMTv-10; 4, M(+)V13; 5, MMTv-7; 6, Mvsrc-9. Relative molecular mass (M_r) markers: a, 116 kilodaltons (kDa) (β -galactosidase); b, 97.5 kDa (phosphorylase b); c, 68 kDa (bovine serum albumin); d, 45 kDa (ovalbumin); e, 29.5 kDa (carbonic anhydrase). (B) Metabolic labeling of pp60^{v-src} in pp60^{v-src}-expressing and control MDCK cell lines. Cells were labeled metabolically with [³⁵S]methionine for 10 h, and pp60 was immunoprecipitated with McAb 327 (46) from cell extracts normalized to total TCA-precipitated counts. The immunoprecipitates were separated by 9 to 15% SDS-PAGE and processed for fluorography. A portion of the resulting fluorogram is presented. The relative levels of [³⁵S]methionine incorporation into pp60 were determined by scanning densitometry. Resulting values were divided by that of MFneoPOP to give relative levels (lanes 1 to 6, respectively of 1:1.6:2.3:2.8:1.5:>8. Lanes, Same as in panel A.

FIG. 4. Formation of individual multicellular (MDCK) cysts in a collagen gel matrix. Cells expressing $pp60^{v-src}$ and control MDCK cells were cultured in a collagen gel matrix for 14 days as described in the text. Cysts were photographed with phase contrast optics. a, MFneoPOP (control); b, MMTv-7; c, d, and e, examples of MMTv-10. Arrows in panel a show the profile of epithelial monolayer in focus; arrows in b through e show cell processes (spikes). At higher magnification the spikes in panels d and e included cell nuclei, which indicates that entire cells have been displaced from these cysts (not shown). Bars, 50 μ m.

that of the MFneoPOP control cells is consistent with the expression of active $pp60^{v-src}$ (see also references 12 and 55).

Low-level expression of $pp60^{v-src}$ in MDCK cells is nonmitogenic and does not cause anchorage-independent growth. It was noted above that cells of the *Mvsrc* clones that expressed high levels of $pp60^{v-src}$ formed epithelial monolayers as cell-cell contacts became extensive at high cell densities. However, after attaining confluency, these cells overgrew the monolayer in multiple focal areas to form cohesive clusters of cells (polyps) (Fig. 2j). These polyps arose from cells which were shed continuously from the monolayer and had the ability to grow in an anchorage-independent manner in the medium. However, when individual polyps were isolated and replated, the cells attached to the substratum and spread out to form epithelial colonies identical to those in the original passage of cells (data not shown). This indicates that polyps arising from these original *Mvsrc* clones (passages 1 to 3) retained clonal characteristics and did not represent the more highly transformed variants which arose after five to seven passages.

The low- $pp60^{v-src}$ -expressing G418^r clones had doubling times (Table 1) and monolayer saturation densities similar to those of the MFneoPOP control cells (data not shown). However, unlike the *Mvsrc* clones, the MFneoPOP, M(+)-V13, M(-)-VB, MMTv-7, and MMTv-10 clones failed to overgrow the monolayer and form polyps, even when the monolayers were overlaid with 0.33% soft agar (Table 1). Thus, it appears that the low- $pp60^{v-src}$ -expressing G418^r clones exhibit growth properties different from those of the high- $pp60^{v-src}$ -expressing *Mvsrc* clones. This difference was confirmed directly by comparison of the clones in a soft agar

growth assay (Table 1). Cells from the *Mvsrc* clone grew well in soft agar, but all the low- $pp60^{v-src}$ -expressing G418^r clones and the control cells failed to grow. These results demonstrate clearly that low-level expression of $pp60^{v-src}$ in MDCK cells does not cause transformation by the standard criteria of anchorage-independent growth potential and increased cell proliferation.

MDCK cells expressing low levels of $pp60^{v-src}$ exhibit cell adhesion disturbance. A striking characteristic of each low- $pp60^{v-src}$ -expressing G418^r clone was the presence of large numbers of single cells on top of the monolayer (Fig. 2i). These cells appeared to have been shed from the monolayer, indicating that $pp60^{v-src}$ expression disrupts both cell-cell and cell-substratum attachments; this phenomenon is referred to as a cell adhesion disturbance. However, these cells failed to grow in an anchorage-independent manner in the medium and subsequently degenerated. These results demonstrate clearly that the cell adhesion disturbance exhibited by the low- $pp60^{v-src}$ -expressing G418^r clones and the anchorage-independent growth potential exhibited by the high- $pp60^{v-src}$ -expressing *Mvsrc* clones are experimentally dissociable traits.

The acquisition of each of these two traits correlated with different relative levels of kinase activity (Table 1). First, the adhesion disturbance, which manifested as cell shedding, was present in all $pp60^{v-src}$ -expressing clones but not in the control. This adhesion disturbance was elicited even in the M(-)-VB clone, which expressed a relative kinase level 20-fold lower than that of the *Mvsrc*-9 clone (Table 1). Thus, the relative kinase level associated with an adhesion disturbance was equal to or less than that expressed by the M(-)-VB clone. Second, the MMTv-7 clone expressed a relative level of kinase which was six to seven times that of the M(-)-VB clone, but showed no augmentation of growth properties of soft agar suspension (Table 1). The relative kinase levels of M(-)-VB and MMTv-7 defined a minimum range which was associated with altered adhesion properties but not with anchorage-independent growth potential. Third, the *Mvsrc*-9 cells expressed a relative kinase level only three times higher than that of MMTv-7 (Table 1). This implies that between the relative kinase levels expressed by MMTv-7 and *Mvsrc*-9, there is a level beyond which cells acquire anchorage-independent growth potential. Most importantly, MDCK cell adhesion disturbances could be elicited by relative $pp60^{v-src}$ levels much lower than those necessary for acquisition of anchorage-independent growth potential.

Deformation of multicellular cyst architecture in MDCK cells expressing low levels of $pp60^{v-src}$. To determine whether MDCK cells expressing $pp60^{v-src}$ retain the morphogenetic determinants required for the formation of higher-order multicellular epithelial structures, cells were grown in a collagen gel matrix (74). Under these conditions, normal MDCK cells divide clonally to form a spherical cyst composed of a closed monolayer of polarized cells in which the apical membranes face the lumen of the cyst and the basal membranes form a smooth, outer surface exposed to the collagen gel matrix (Fig. 4a; see also Fig. 6a).

Analysis of low- $pp60^{v-src}$ -expressing clones growing in a collagen matrix revealed a significant deformation of cyst architecture (Fig. 4). After 14 days, a clear spectrum of cystic structures became evident. More than 95% of MMTv-7-derived cysts appeared highly disorganized (Fig. 4b) compared with the control (Fig. 4a); the cysts were not spherical and had numerous projections (Fig. 4b). Cysts derived from M(+)-V13 and MMTv-10 formed spherical cysts, of which

FIG. 5. Formation of disk-shaped multicellular (MDCK) cysts grown in a collagen gel matrix. $pp60^{v-src}$ and control MDCK cells were grown on and then overlaid with a collagen gel matrix to produce disk-shaped cysts as described in the text. Cysts were photographed with phase contrast optics (a and c). They were subsequently fixed and embedded in Plexiglas, and thin sections ($3\ \mu\text{m}$) were prepared. The sections were stained with toluidine blue and photographed with bright-field optics (b and d). a and b, MFneoPOP (control); c and d, MMTv-7. Arrows in panel a show control cysts with smooth contours, without spikes; arrows in panel c show cysts with jagged edges with multiple spikes (bar, $100\ \mu\text{m}$). Small arrows in panels b and d show an intact epithelial monolayer; large arrows in panel d show four displaced cells at the edge of an MMTv-7 cyst (bar, $10\ \mu\text{m}$).

~50% had cell processes (spikes) projecting from the basal surface of the cyst into the collagen gel matrix; some spikes appeared to be entire cells (Fig. 4c, d, and e; also see below). The cysts derived from M(-)VB and MFneoPOP formed spherical cysts with no spikes (for example, Fig. 4a).

These results show that deformation of cyst architecture was exhibited only in $pp60^{v-src}$ -expressing clones. Significantly, the ability of these clones to form normal-appearing cysts correlated with the relative level of kinase activity. The MMTv-7 clone formed highly deformed cysts and expressed the highest relative kinase levels of the clones tested (16-fold above the control); M(-)VB formed normal-appearing cysts (not shown) and expressed the lowest relative $pp60^{v-src}$ kinase levels; M(+)V13 and MMTv-10 showed intermediate deformation of cyst architecture and expressed relative kinase levels intermediate between those of M(-)V13 and MMTv-7.

But if $pp60^{v-src}$ causes abnormal cyst growth and organization, why did only limited regions of the cyst wall bear spikes? A trivial explanation is that many small spikes existed but were not apparent by phase contrast optics. Analysis of serial thin sections of plastic-embedded cysts did allow detection of additional areas of spike formation, but even at this resolution it was apparent that many of the MMTv-10- and M(+)V13-derived cysts did not bear spikes (data not shown).

The nonuniform distribution of this trait in cysts, together with the fact that open monolayers exhibited disturbed cell adhesion (see above), suggested to us that $pp60^{v-src}$ may act in a permissive way to make the cells within open or closed

monolayers susceptible to displacement by independent physical forces within the epithelial monolayer. We predicted that if such physical force were preferentially exerted in one region of the cyst wall, the trait might manifest uniformly in this region. Therefore, the assay was modified to yield only flat, disk-shaped cysts which should have maximal tension at the periphery, where the epithelium bends acutely (see Materials and Methods) (Fig. 5). MFneoPOP, MMTv-10, and MMTv-7 were compared in this assay. MFneoPOP cells formed oval cysts with smooth contours and no spikes (Fig. 5a); however, all MMTv-10 and MMTv-7 cysts bore jagged edges (for example, Fig. 5c). The morphology and organization of cells at the edge of these flat cysts were analyzed in detail in serial thin sections of plastic-embedded cysts. MFneoPOP cysts had a smooth, contoured disk shape, and the closed monolayer of cells exhibited no evidence of disorganization even at the edges, where the monolayer bent acutely (Fig. 5b). On the other hand, the edges of the MMTv-7-derived cysts exhibited considerable deformation where numerous cells were displaced from the basal aspect of the monolayer into the collagen gel matrix (Fig. 5d). The deformation of the cyst was manifested uniformly and exclusively at the edges; the remainder of the cyst maintained a highly organized monolayer of cells identical in shape to the control (compare Fig. 5b and d). Presumably cells were displaced at the periphery of these flat cysts because cell division expanded the closed monolayers in the horizontal plane, creating maximal shear on the equatorial cells (note that the rate of cell growth was the same for all clones [Table 1]). Control

FIG. 6. Ultrastructure of multicellular pp60^{v-src}-expressing and control MDCK cell cysts grown in a collagen gel matrix. MDCK cells were cultured in a collagen gel matrix for 14 days and then processed for analysis by transmission electron microscopy. (a and b) Low-power survey electron micrographs showing sections of multicellular cyst walls from control (a) and MMTv-10 (b) MDCK cells. The apical aspect of the cyst wall faces the lumen (top), and the basal aspect of the cyst wall faces the collagen matrix (bottom) in each picture. Arrows in panel a show the close apposition of lateral membranes between adjacent cells in the MDCK (control) cyst wall. Small arrows in panel b show separation of lateral membranes between adjacent cells in the MMTv-10 cyst wall. Large arrows in b indicate two cells which are partially displaced from the basal aspect of the MMTv-10 cyst wall. These cells correspond to a small spike visualized by phase contrast microscopy (see Fig. 4). (c to g) High-magnification electron micrographs showing junctional complexes and cell-cell contacts between the lateral membranes of adjacent cells within cyst walls. (c and d) MDCK (control) cells; (e and f) MMTv-10 cells; (g) Mvsrc-9 cells. The brackets in panel c show close apposition of lateral membranes between adjacent MDCK (control) cells. Arrows in panels e and f show the scalloped appearance of the enlarged intercellular spaces between adjacent MMTv-10 cells. Ap, Apical plasma membrane; bl, basal plasma membrane; cp, coated pit; des, desmosome; N, nucleus; za, zonula adherens; zo, zonula occludens. Bars: 1 μ m (a and b), 0.25 μ m (c to g).

cysts were cohesive enough to withstand this force, but cysts expressing pp60^{v-src} could not, resulting in the displacement of cells at the edge.

Selective uncoupling of the junctional complex in MDCK cells expressing low levels of pp60^{v-src}. To investigate the degree of cell-cell contact in control and low-pp60^{v-src}-expressing cells, cysts were analyzed by transmission electron microscopy (Fig. 6). The closed epithelial monolayer that formed the wall of control cysts exhibited a high degree of intercellular adhesion (Fig. 6a). The lateral membranes of adjacent cells were closely apposed, with rare instances of an intercellular space greater than 50 nm (Fig. 6a and c). The zonula occludens appeared at the boundary of the apical and lateral membranes on the luminal side of the cyst and was characterized by the very close apposition of adjacent cell

membranes (Fig. 6c and d). Invariably, the zonula adherens was located $\sim$ 100 nm below the zonula occludens and was characterized by an intercellular gap of $\sim$ 20 nm (Fig. 6c and d). Numerous desmosomes with associated tonofilament bundles were located randomly on the lateral membranes, but were invariably excluded from the apex of the membrane in the region of the zonula occludens and zonula adherens (Fig. 6a and d).

Electron microscopy of pp60^{v-src}-expressing cysts showed that the cells were morphologically polarized, with numerous microvilli on the (apical) membrane facing the cyst lumen, similar to the control cysts (compare Fig. 6a and b). However, there were striking abnormalities in the ultrastructure of lateral membranes between adjacent cells (Fig. 6b, e, f, and g). First, significant intercellular spaces were present

FIG. 7. Indirect immunofluorescence of pp60^{v-src} in MDCK cells. Indirect immunofluorescence assays were performed on MDCK cells that had been fixed with 1.75% formaldehyde and permeabilized with 0.5% (vol/vol) Triton X-100 in PBS. The first antibody was either a McAb directed against pp60^{v-src} (b to j) or a nonimmune mouse serum (a). The second antibody was rhodamine-conjugated goat anti-mouse IgG. a, MMTv-10; b, MFneoPOP (control); c and d, MMTv-10; e to j, Mvsrc-9. Large arrows in panels e, g, h, i, and j show staining of regions of cell-cell contact. Small arrows in panel f and j show staining of focal adhesion plaques. All photographs are presented at the same exposure. All panels but f are presented at the same magnification. Bars, 25 μ m.

along the complete length of the lateral membranes; the spaces formed a scalloped profile with a maximum width of ~ 110 to 175 nm between the separated membranes (Fig. 6b, e, and f). Second, the zonula adherens appeared to be either disrupted or, more frequently, completely missing, at least at the ultrastructural level (Fig. 6e, f, and g). Third, coated vesicles (Fig. 6e) or desmosomes (Fig. 6g) were frequently observed in the region of the membrane normally occupied by the zonula adherens, further indicating a disruption in the formation of the zonula adherens (compare with normal cells, Fig. 6d). These lesions were present in low- and high-pp60^{v-src}-expressing clones.

These results indicate that the structure of the junctional complex is selectively uncoupled in pp60^{v-src}-expressing cells: the zonula adherens is disrupted, but the zonula occludens and desmosomes are retained. The selective disruption of the junctional complex elicited by pp60^{v-src} expression differs from the effects of nonspecific agents such as phorbol esters, which disrupt all contacts except the desmosomes (69), and chelators of Ca^{2+} , which have been shown to disrupt the zonula adherens and desmosomes (70). However, the effect of pp60^{v-src} shown in this study does resemble alterations in MDCK cells exposed to anti-Arc-1 antibodies, which recognize a uvomorulinlike canine cell adhesion molecule (1, 33). The fact that pp60^{v-src} expression disrupts regions of cell-cell contact which are known to depend normally on uvomorulinlike cell adhesion molecules raises the possibility that pp60^{v-src} may regulate, directly or indirectly, the function of this type of cell adhesion molecule (see below).

The disruption of the zonula adherens portion of the junctional complex and regions of cell-cell contact in the pp60^{v-src} clones was evident not only in regions of the cyst wall in which cells were being displaced into the collagen matrix, but also in regions in which the integrity of the monolayer was intact (Fig. 6b). In fact, the M(-)VB cells, which expressed low pp60^{v-src} levels, showed these ultrastructural defects even though M(-)VB cysts did not form spikes (data not shown; Table 1). Apparently, the level of expression of pp60^{v-src} in the M(-)VB clone is sufficient to

cause low-level disruption of cell-cell contacts which is detectable by electron microscopy and to lead to cell shedding from densely packed open monolayers (Fig. 3). However, the level of pp60^{v-src} expression in M(-)VB is apparently below a level necessary to allow cell displacement from cyst walls into the collagen gel matrix (Table 1). Our interpretation of these observations is that each low-pp60^{v-src}-expressing clone [MMTv-7, MMTv-10, M(+)V13, and M(-)VB] has a different degree of reduction in cell-cell adhesion throughout the cell monolayer that forms the cyst wall and that this predisposes the cysts to different degrees of deformation.

Areas of MDCK cell-cell contact are enriched with immunoreactive pp60^{v-src}. Our analyses of both the overall architecture and ultrastructure of pp60^{v-src}-expressing cysts indicate that expression of this tyrosine kinase in MDCK cells results in the disruption of the selective regions of the membrane involved in cell-cell contact. Consequently, we sought to determine whether the subcellular distribution of pp60^{v-src} correlates with this apparent target region in the cell. Immunofluorescence assays were performed on MFneoPOP, MMTv-10, MMTv-7, and Mvsrc-9 cell lines with a McAb raised against pp60^{v-src}; all micrographs are presented at the same exposure (Fig. 7). MFneoPOP cells exhibited a faint, diffuse staining pattern, presumably reflecting the distribution of endogenous pp60^{c-src} (Fig. 7b). Cells derived from MMTv-10 exhibited a more intense diffuse staining pattern, but in addition exhibited consistent staining of the cell periphery in the region of cell-cell contact (Fig. 7c and d). Similar staining of the cell periphery and focal adhesion plaques was found also in the MMTv-7 cell line (data not shown). Mvsrc-9 cells exhibited a much more pronounced staining pattern, consistent with the higher expression of pp60^{v-src} in these cells than in MMTv-10 (and MFneoPOP; see Table 1). pp60^{v-src} staining was observed in two distinct regions in Mvsrc-9 cells (Fig. 7e to j). First, single cells and cells in colonies exhibited intense staining of patches on the ventral surface of the membrane at the cell periphery (Fig. 7f); similar patches stained with an antiserum raised against canine vinculin and probably represented

focal adhesion plaques (data not shown). Second, and most significant, regions of cell-cell contact stained intensely; this was apparent in colonies of cells (Fig. 7g to j) as well as in the region of contact between cells at low cell density (Fig. 7e). Towards the centers of colonies, the pattern of staining appeared to completely circumscribe each cell (Fig. 7g to j).

These morphological analyses demonstrate clearly that in pp60^{v-src}-expressing clones there is a selective disruption of areas of cell-cell contact which, at least at the resolution of the light microscope, are highly enriched with pp60^{v-src}. These results suggest strongly that the lateral membrane of adjacent MDCK cells is a target for pp60^{v-src} localization and function.

Concluding remarks. We used MDCK cells to study the biological activities of pp60^{v-src} because of their intrinsic capacity to form higher-order epithelial structures which show a striking structural and functional resemblance to renal tubules in vivo. MDCK cells are already highly differentiated and do not undergo a sequential inductive process to form multicellular structures, which characterizes epithelial morphogenesis in situ (2, 24). This is important, because the morphogenetic trait detected in these multicellular epithelial structures may be attributed to the expression of the introduced *v-src* gene and not to independent inductive factors. These features of MDCK cells have allowed, for the first time, the detection of a nonmitogenic biological trait which results from levels of pp60^{v-src} that are below those which lead to transformation, as judged by the absence of anchorage-independent growth potential and increased cell proliferation. In contrast, cultured rodent fibroblasts which express pp60^{v-src} levels below the threshold for transformation do not acquire a phenotype that is distinguishable from control fibroblasts (35).

The results of our studies demonstrate that pp60^{v-src} expression perturbs lateral membrane contacts and the formation of the zonula adherens between adjacent MDCK cells. However, it is unclear whether pp60^{v-src} directly affects the zonula adherens or whether the zonula adherens is disrupted secondarily as a consequence of lateral membrane separation. The possibility that pp60^{v-src} may act directly on the zonula adherens is supported by the fact that this structure has many similarities with focal adhesion plaques, which are known to be a target of pp60^{v-src} action in fibroblasts (56). Both structures are discrete plasma membrane domains to which subcortical actin filaments bind (19, 29, 65, 71), and both structures are rich in α -actinin and vinculin (19). It will be important in future studies to determine whether the distribution and function of these proteins are affected at the zonula adherens in pp60^{v-src}-expressing MDCK cells; our preliminary studies indicate that the subcortical band of actin normally associated with the zonula adherens is disrupted in the low-pp60^{v-src}-expressing clones (our unpublished results).

Significantly, this ultrastructural perturbation coincides with a distinctive trait, disturbed intercellular adhesion. This trait is manifested as cell shedding from monolayers and cell displacement from mechanically stressed regions of multicellular epithelial structures. However, despite a clear disruption of the zonula adherens and the separation of large portions of adjacent lateral membranes, the two-dimensional relationship between the majority of the cells within the cyst wall are maintained. These results provide a new insight into the role of cell-cell adhesion components, including the zonula occludens, zonula adherens, desmosomes, and specific cell adhesion molecules, in establishing and maintaining the three-dimensional architecture of higher-order epithelial

structures such as cysts or tubules (14, 17, 54, 63, 65, 68, 73). In addition, our results indicate that the zonula occludens and desmosomes are not subject to the same regulatory mechanisms controlling adhesion of the zonula adherens or regions of the lateral membrane excluded from these junctional complexes.

The trait elicited by pp60^{v-src} in this in vitro system appears to resemble certain morphogenetic phenomena in vivo. First, temporal and spatial variation of epithelial tension and plasticity are thought to be critical for the formation of three-dimensional tubular or cystic tissue patterns in vivo. This would require modulation of cell-cell adhesion without disrupting the two-dimensional organization of the epithelial monolayer (14, 30, 37, 54, 65, 68, 72, 73). Second, epithelial cells are known to emigrate from the basal aspect of epithelia during development to give rise to secondary tissue structures (27, 57; for reviews, see references 14, 28, and 68). This fundamental pattern of histogenesis is particularly prominent in the neuroepithelia, which give rise to the mantle layer in the developing central nervous system and to the neural crest cells of the developing peripheral nervous system (16, 26, 43, 59). These histogenetic processes would also require discrete adjustments of cell-cell adhesion among the cells within these epithelial structures.

The fact that pp60^{c-src} expression has been detected in postmitotic differentiating cells that are undergoing dynamic changes in their spatial organization (for references, see Introduction) raises the question of whether pp60^{c-src} plays a role in modulating cell adhesion and tissue plasticity, similar to the action of pp60^{v-src} in MDCK cells. In this respect, it should be noted that we have consistently detected endogenous pp60^{c-src} expression in control MDCK cells that do not exhibit cell adhesion disturbance. If pp60^{c-src} has the potential to elicit this trait, then its activity must be restricted in intact MDCK cells (see also reference 12). However, it may be significant that elevated expression of a variant pp60^{c-src} (coding sequence derived from pHb-5 [45]) in MDCK cells produces a nontransformed phenotype which is similar to that seen in the low-pp60^{v-src}-expressing cell lines; viruses containing the variant pp60^{c-src} do not cause a detectable phenotype in fibroblasts (Warren and Nelson, manuscript in preparation). The fact that a nononcogenic variant of pp60^{c-src} and pp60^{v-src} have a similar biological action on multicellular epithelial structures, without causing uncontrolled cell proliferation, suggests that these tyrosine kinases have retained the potential to act on morphogenetic determinants that may be controlled normally by a related cellular tyrosine kinase(s).

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