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Adhesion plaques of Rous sarcoma virus-transformed cells contain the *src* gene product

(interference-reflection microscopy/pp60^{src} phosphoprotein/immunofluorescence microscopy/cytoskeleton/cellular "feet")

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ABSTRACT **Another intracellular location of the Rous sarcoma virus (RSU) *src* gene product (pp60^{src}) has been detected within RSV-transformed cells by indirect immunofluorescence. By using rabbit anti-tumor serum specific for pp60^{src}, a speckled pattern of fluorescence was found on the ventral surface of RSV (Schmidt–Ruppin strain)-transformed normal rat kidney cells. Several tests indicated that this pattern was specific for pp60^{src}. In addition, interference-reflection microscopy was used to visualize cellular adhesion plaques, which are the points at which cells attach to the substratum. Simultaneous immunofluorescence and interference-reflection microscopy indicated that the speckles of pp60^{src} fluorescence corresponded exactly to the adhesion plaque structures. The presence of pp60^{src} within the adhesion plaques was further demonstrated by indirect immunofluorescence on isolated adhesion plaques that remained bound to glass after removal of the cells. pp60^{src} also was observed in adhesion plaques of RSV-transformed chicken embryo fibroblasts (CEF) and mouse fibroblasts, as well as CEF infected with the temperature-sensitive RSV mutant tsNY68 and grown at permissive temperature. At nonpermissive temperature, pp60^{src} was not detectable in adhesion plaques of the tsNY68-infected CEF. Adhesion plaques serve as focal points of microfilament bundle attachment, and these results suggest that pp60^{src} interacts directly with cellular cytoskeletal components.**

Rous sarcoma virus (RSV) has been a valuable tool for examining the mechanism of neoplastic transformation because it transforms a broad spectrum of avian and mammalian cells. The transforming function of the virus is encoded in a single gene called *src* (1) and, so far, only a single transforming protein has been identified as the *src* gene product (2). This protein is a 60,000 M_r phosphoprotein (termed pp60^{src}) (3–5) containing two major phosphorylation sites per molecule (6). One site is probably regulated by a cyclic AMP-dependent protein kinase, whereas the second site may involve autophosphorylation through a unique kinase activity associated with pp60^{src}. This kinase activity phosphorylates substrate proteins on tyrosine residues (7). That the kinase activity is indeed encoded in the *src* gene has been suggested by its invariable association with pp60^{src} (8, 9), its temperature dependence in T-class mutants (4–6, 8, 9), and its copurification with partially purified pp60^{src} (10, 11). These results all suggest that transformation in this system may result from mechanisms involving phosphorylation by pp60^{src}.

It is not known whether pp60^{src} interacts with many cellular target sites or whether a monofunctional pp60^{src} acts at a single target to effect transformation and the numerous alterations associated with transformed cells (12). Transformation by RSV, however, is known to disrupt microfilament bundles, and therefore a cytoplasmic target for pp60^{src} may directly or indirectly control the organization of microfilament bundles within the cytoplasm (13, 14). This possibility is compatible with studies that have localized pp60^{src} to various cytoplasmic compartments (15–17); however, the functional significance of these locations in maintaining the transformed phenotype is not understood.

This paper presents results of further efforts to analyze the intracellular location of pp60^{src} by indirect immunofluorescence. In addition, a technique called interference-reflection microscopy has enabled the direct visualization of cellular plaques that are in contact with the substrate on which the cells grow (18, 19). These contact points are termed "adhesion plaques" or, more figuratively, "feet" and represent focal points of stress fiber organization (19–21). The results in this paper indicate that a functional pp60^{src} is associated with the cellular adhesion plaques in RSV-transformed cells.

METHODS AND MATERIALS

Cells and Viruses. Chicken embryo fibroblasts (CEF) and SR-NRK cells (RSV$^-$ transformed normal rat kidney cells, clone A4B5G4) were as described (16, 22). The Schmidt–Ruppin strain of Rous sarcoma virus subgroup D (SR-RSV-D) was from a recently cloned laboratory stock. A mutant of SR-RSV-A, designated tsNY68, was obtained from Mike Weber (Univ. of Illinois). A line of SR-RSV-D-transformed BALB/c cells (SR-BALB) was obtained from S. Aaronson (National Institutes of Health) and recloned by end point dilution.

Immunofluorescence. Cells were grown on ethanol-sterilized glass coverslips, fixed 20 min in 4% (wt/vol) paraformaldehyde in phosphate-buffered saline (P_i/NaCl), rinsed in P_i/NaCl, and treated 3 min in 0.2% Triton X-100 in P_i/NaCl to make cells permeable. The sera and remainder of the technique were as described (16). All sera were absorbed just prior to use with 250 μg of unlabeled SR-RSV-D, which was then removed by centrifugation. For some experiments the cells were removed from the coverslips by an unpublished detergent treatment method that leaves the adhesion plaques attached to the glass. Briefly, cells were extracted with 0.2% Triton X-100 on ice, fixed in paraformaldehyde, then gingerly blown from the coverslip under a stream of P_i/NaCl.

Interference-Reflection Microscopy. The method of Curtis (18) was followed for setting the Zeiss Universal Photomicroscope for recording interference-reflection images. Illumination was from a HBO 200 W/4 high-pressure mercury lamp. The lamp field stop was closed and the green (BP 546) rhodamine filter was inserted into the optic path of the epifluorescence condenser III RS. A polarizing filter was used in the barrier filter slide to reduce the intensity of illumination, and the image was observed through a ×40 oil immersion objective.

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Abbreviations: RSV, Rous sarcoma virus; SR-RSV-D, Schmidt–Ruppin strain of RSV subgroup D; CEF, chicken embryo fibroblasts; SR-NRK, normal rat kidney cells transformed with SR-RSV-D; pp60^{src}, the 60,000 M_r phosphoprotein product of the RSV transforming gene; P_i/NaCl, phosphate-buffered saline.

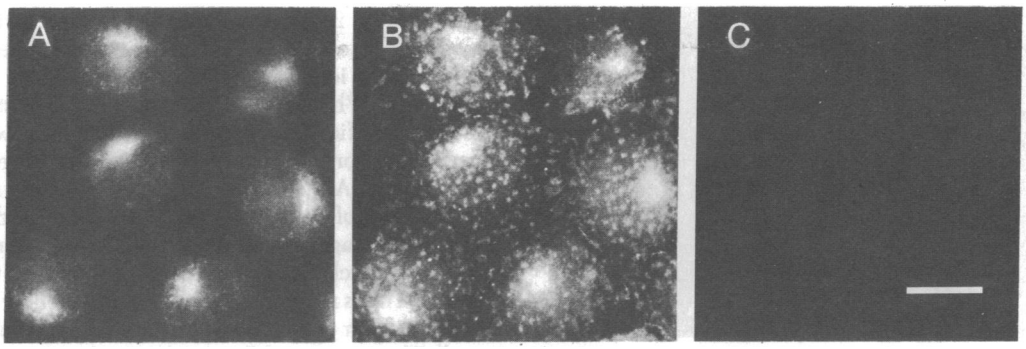

FIG. 1. Patterns of pp60^{src} localization within SR-NRK cells detected by indirect immunofluorescence. SR-NRK cells were fixed and stained with rabbit antibodies to pp60^{src} and rhodamine-conjugated goat antiserum to rabbit IgG. (*A*) Fluorescence pattern seen with focal plane set at mid-nuclear level or slightly higher, weak cytoplasmic and prominent perinuclear spot fluorescence evident. (*B*) Fluorescence pattern seen in same field of cells with focal plane set near ventral surface of cells; note speckled pattern of fluorescence. (*C*) SR-NRK cells allowed to react with normal rabbit serum in place of anti-tumor serum, focus near ventral cell surface. Bar in *C* = 20 μm.

Photographic Techniques. Rhodamine fluorescence was recorded on Kodak Tri-X pan film rated at ASA 1600 and developed in Diafine (Acufine, Chicago, IL). Interference-reflection images were recorded on Kodak Panatomic X film rated at ASA 128 and developed in Kodak Microdol X. All photographs were printed on high-contrast paper. Interference-reflection images are often presented as composites due to the limited field of view resulting from the necessity of closing the lamp field stop to increase image contrast.

RESULTS

Patterns of pp60^{src}-Specific Fluorescence in SR-NRK Cells. The rabbit anti-RSV-tumor serum used in these studies has been characterized by immune precipitation and immunofluorescence, and it appears to be specific for pp60^{src} when preabsorbed with unlabeled virus (16, 22, 23). The results in Fig. 1 demonstrate some of the patterns of pp60^{src}-specific fluorescence detectable within SR-NRK cells by using this preabsorbed antitumor serum. The fluorescence patterns that one sees are greatly dependent on where the plane of focus is set within the cells. When the focal plane rests about midnuclear level or higher, a characteristic perinuclear spot of fluorescence is detectable. This spot is cytoplasmic but sometimes, depending upon the geometry of observation, appears flattened against the outer nuclear membrane (Fig. 1*A*). A weak diffuse cytoplasmic fluorescence also can be seen within the SR-NRK cells. In addition, pp60^{src}-specific fluorescence is usually found at gap-junctions between SR-NRK cells (16, 17); however, junction formation had not yet occurred in the relatively sparse culture shown in Fig. 1. Most of these fluorescence patterns for pp60^{src} have been observed previously (15–17, 22); however, the perinuclear distribution of pp60^{src} was not detected by immunoferritin electron microscopy (17).

In contrast to the results shown in Fig. 1*A*, a speckled pattern of fluorescence was seen in the same SR-NRK cells shown in Fig. 1*A* when the focal plane was shifted to the region of cell-substrate attachment (Fig. 1*B*). This speckled pattern was superimposed on the perinuclear spot pattern, and was not detectable in Fig. 1*A* due to the shallow depth of field of the objective lens and the relatively weak intensity of the speckles. This pattern was not peculiar to the rabbit anti-tumor serum, because antiserum from a marmoset bearing an RSV-induced

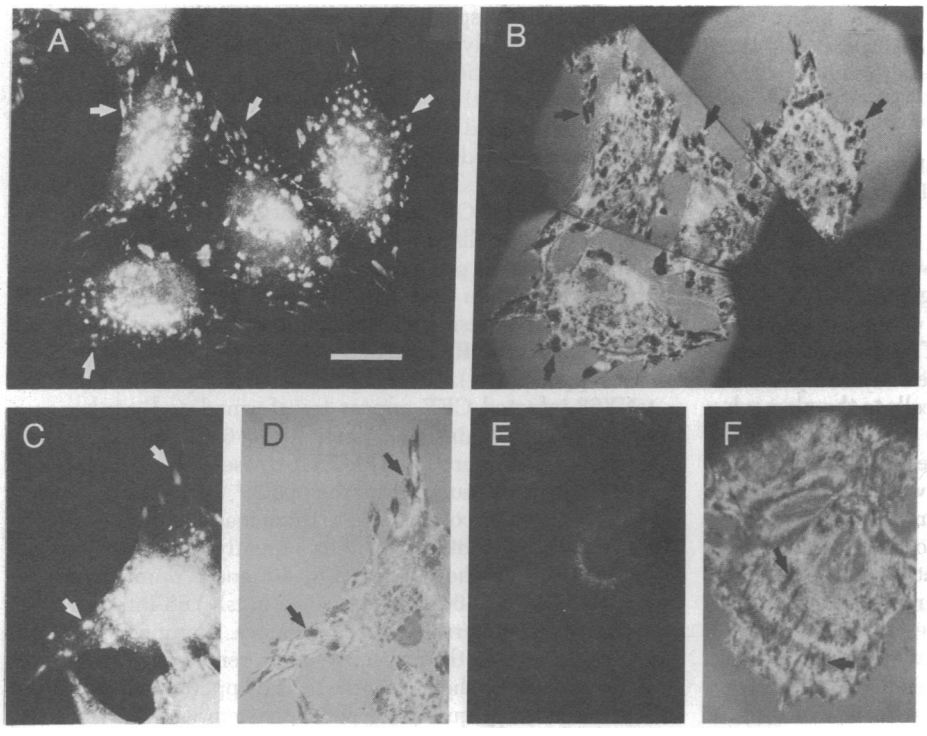

FIG. 2. Comparison of speckled pattern of pp60^{src} fluorescence with cellular adhesion plaques. Cells were fixed and pp60^{src} was stained by indirect immunofluorescence. The pp60^{src} fluorescence at the ventral cell surface was photographed and the adhesion plaques (dark spots) on the same field of cells were then recorded by interference reflection microscopy. (*A* and *C*) pp60^{src} fluorescence on SR-NRK cells; (*B* and *D*) interference reflection image of cells shown in *A* and *C*, respectively. Note that *B* is a composite of three interference reflection fields. (*E*) Fluorescence on NRK cells allowed to react with antiserum to pp60^{src}; (*F*) interference reflection image of NRK cells shown in *E*. Arrows emphasize prominent fluorescent speckles and their corresponding adhesion plaques. Bar in *A* = 20 μm.

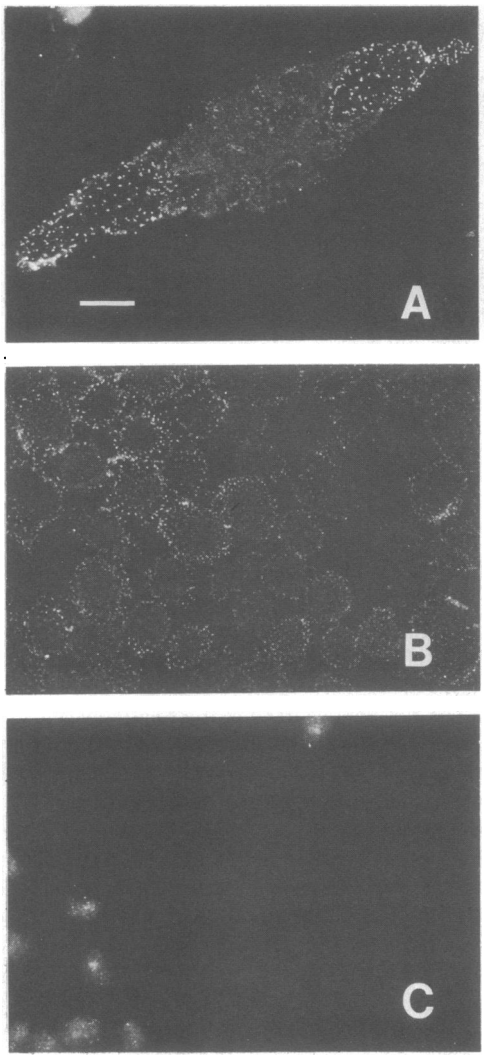

FIG. 3. Detection of pp60^{src} fluorescence in coverslip-bound adhesion plaques after removal of SR-NRK cells. SR-NRK cells, grown on glass coverslips, were removed by a detergent treatment method that left the adhesion plaques bound to the coverslip. The adhesion plaques were fixed in 4% paraformaldehyde, rinsed, and stained with anti-tumor serum to pp60^{src} by indirect immunofluorescence. (*A*) The adhesion plaques from a single giant SR-NRK cell stained with anti-tumor serum. (*B*) A field of SR-NRK adhesion plaques stained with anti-tumor serum. (*C*) A field of SR-NRK adhesion plaques allowed to react with normal rabbit serum. Note that a few nuclei are present in *C* but they are totally absent from *A* and *B*. Bar in *A* = 20 μm.

tumor has given similar results (not shown). Normal rabbit serum did not stain the SR-NRK cells (Fig. 1*C*).

Comparison of the Speckled Pattern of pp60^{src} Fluorescence with Cellular Adhesion Plaques. The speckled pattern of pp60^{src} specific fluorescence was situated within a plane on or near the point of attachment of these cells to the glass substrate. This suggested that perhaps some component of cell-substrate attachment at the ventral surface of these cells could be associated with pp60^{src}. This possibility was explored further by utilizing an optical technique that permits the visualization of the points at which the cell attaches to the glass substrate (18–20). This optical technique is called interference-reflection microscopy, and the cellular contact points detected by this method have been called adhesion plaques or "feet."

The simultaneous use of interference-reflection microscopy and immunofluorescence microscopy enabled the direct comparison of pp60^{src} fluorescent speckles at the ventral surface of SR-NRK cells with adhesion plaques on the same cells. The results in Fig. 2 *A* and *C* show the speckled pattern of pp60^{src} fluorescence on SR-NRK cells. The interference-reflection images of the identical fields are shown in Fig. 2 *B* and *D*, respectively. White arrows point to characteristic speckles of pp60^{src}-specific fluorescence, and black arrows point to respective regions of cells recorded by interference-reflection microscopy. Adhesion plaques can be seen as dark spots. Comparison of the respective fluorescence and interference-reflection images, using the arrows to aid alignment, reveals that each speckle of fluorescence corresponds to an adhesion plaque. This is most obvious at the cell periphery but is also seen directly under cells, where some adhesion plaques appear very small. The intensity of fluorescence within the adhesion plaques did not always correlate with the size of the adhesion plaque. This was again most evident directly under the cells and may be due to the fact that some plaques were in the process of formation while others were being dissolved at the time of fixation. The speckled pattern of pp60^{src} fluorescence was specific for SR-NRK cells and not detectable on NRK cells (Fig. 2*E*) even though prominent adhesion plaques could be seen (Fig. 2*F*).

To further confirm that the pp60^{src} was situated in the cellular adhesion plaques, cells were removed from the glass coverslips by using a detergent treatment method that leaves the "feet" behind, still attached to the glass coverslip. The SR-NRK "feet" still stained brightly when tested for pp60^{src} by immunofluorescence, using the rabbit anti-tumor serum (Fig. 3 *A* and *B*). The intensity, however, was less than that seen on whole cells, due perhaps to partial extraction of pp60^{src} during the isolation procedure. Normal serum did not stain these "feet" well (Fig. 3*C*), and likewise anti-tumor serum did not stain NRK "feet" (see Fig. 2 *E* and *F*).

Adhesion Plaques and pp60^{src} in Other RSV-Transformed Cells. The association of pp60^{src} fluorescence with cellular adhesion plaques was not limited to the epithelioid SR-NRK cells. Fluorescent adhesion plaques also were observed in RSV-transformed fibroblasts, but sometimes with more difficulty due to more intense cytoplasmic pp60^{src} fluorescence. The results in Fig. 4 *A* and *B* show pp60^{src} fluorescence and the exact interference-reflection image of SR-RSV-D-transformed CEF. The fluorescent spots, illuminating the location of pp60^{src}, correspond exactly to adhesion plaques as recorded by the interference-reflection technique. Furthermore, on focusing at different planes throughout these cells it was apparent that ruffled or blebbed structures containing pp60^{src} fluorescence were often situated directly above many adhesion plaques (not shown). The results in Fig. 4 *C* and *D* also demonstrate that, like SR-NRK and SR-CEF, adhesion plaques of SR-RSV-D-transformed mouse cells also contain pp60^{src}.

Association of pp60^{src} with Adhesion Plaques in tsNY68-Infected Chicken Cells. The association of an active pp60^{src} with cellular adhesion plaques was examined in CEF infected with a mutant of RSV (tsNY68) encoding a temperature-sensitive pp60^{src} (24). At the permissive temperature (35°C), tsNY68-infected CEF appear transformed and synthesize a pp60^{src} that contains kinase activity (4–6, 9, 15). At the nonpermissive temperature (41.5°C), these same cells appear morphologically normal and the pp60^{src}, even though synthesized in amounts comparable to those made under permissive temperature conditions, exhibits a greatly reduced kinase activity (5, 6, 15). The results in Fig. 4 *E* and *G* demonstrate the distribution of pp60^{src} fluorescence in tsNY68-infected CEF maintained at permissive temperature. Comparison of the fluorescence with the respective interference-reflection images (Fig. 4 *F* and *H*) shows that the tsNY68 pp60^{src} was associated with adhesion plaques at the permissive temperature. However, after the tsNY68-infected CEF had grown at nonpermissive

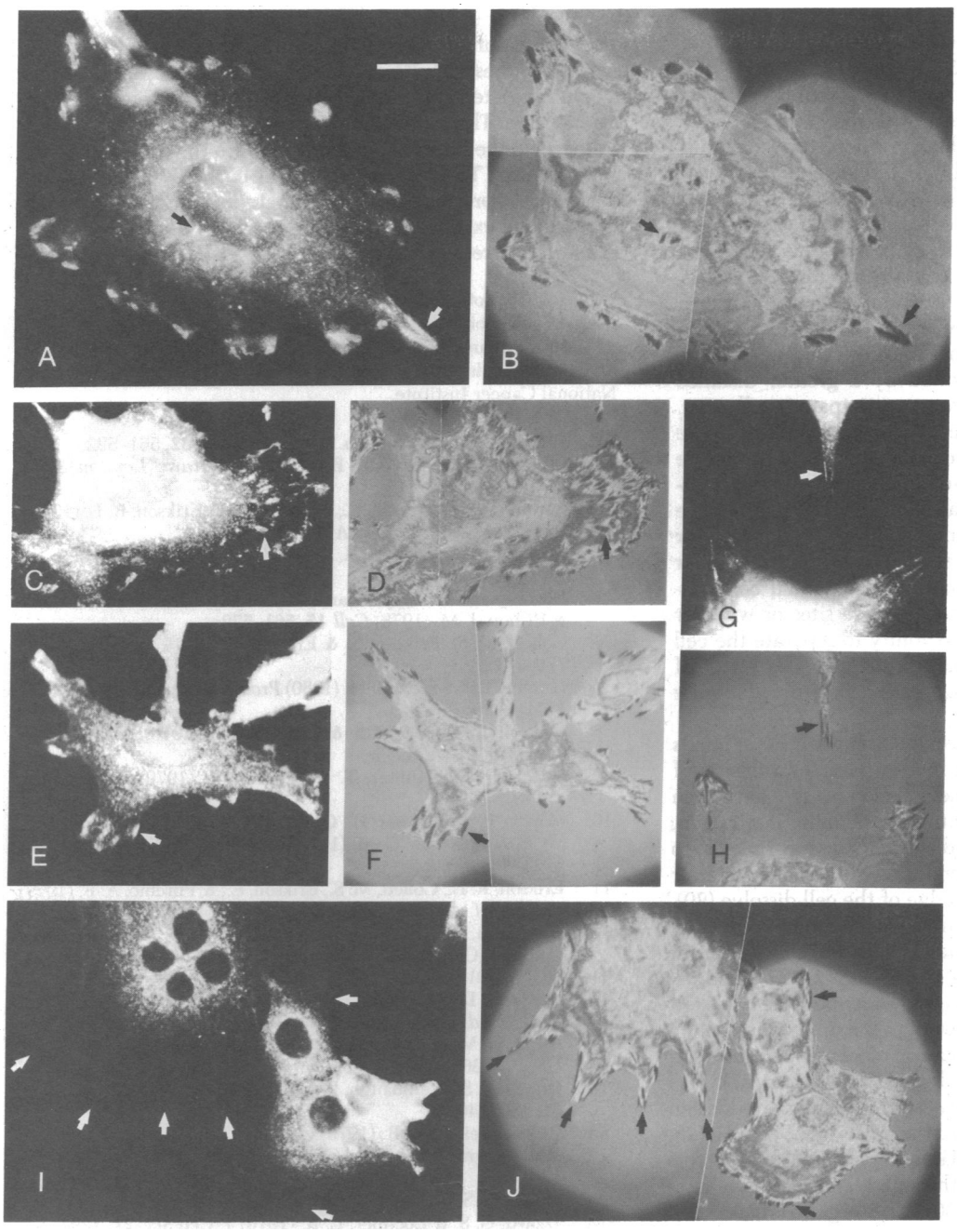

FIG. 4. Comparison of pp60^{src} fluorescence and adhesion plaque location in various RSV-transformed cells. Cells were fixed and stained for pp60^{src} by indirect immunofluorescence. The pp60^{src} fluorescence was photographed and then the interference reflection image of the same field of cells was recorded to show the adhesion plaques or "feet." (*A* and *B*) SR-CEF, fluorescence and "feet," respectively; (*C* and *D*) SR-BALB, fluorescence and "feet," respectively; (*E* and *F*) tsNY68-infected CEF grown at permissive temperature (35°C), fluorescence and "feet," respectively; (*G* and *H*) same as previous pair; (*I* and *J*) tsNY68-infected CEF grown at nonpermissive temperature (41.5°C), fluorescence and "feet," respectively. Arrows emphasize a few "feet" and corresponding location in the fluorescence photographs. Bar in *A* = 20 μm.

temperature for at least 24 hr, the localization of pp60^{src} within adhesion plaques was greatly reduced or totally eliminated (compare Fig. 4 *I* with *J*). In agreement with the results of others (5, 15), pp60^{src} was detectable at the nonpermissive temperature but situated only in what appeared to be the soluble cytoplasm even though conspicuous adhesion plaques were visible in the mutant-infected cells maintained at nonpermissive temperature.

DISCUSSION

The results in this paper bring together two observations pertinent to the mechanism of RSV-induced transformation. These observations were made through the combined use of indirect immunofluorescence microscopy and interference-reflection microscopy. First, by using rabbit anti-tumor serum and immunofluorescence an intracellular location of pp60^{src} in addition to previously observed cytoplasmic locations of pp60^{src} has been found. This location was visualized as a speckled pattern of fluorescence on the ventral subsurface of RSV-transformed cells. This speckled pattern appeared specific for pp60^{src} and was not detected in uninfected cells, and previous results suggested that it was not on the external cell surface (16, 17). Therefore, pp60^{src} was probably contained on the cytoplasmic side of specific structures located on the ventral cell surface. Second, the speckled pattern of pp60^{src} had been observed previously (see figure 2g in ref. 16); however, the significance of this localization was not understood until comparison was made with cellular adhesion plaques visualized by interference-reflection microscopy. This analysis demonstrated that the speckles of pp60^{src} detected by immunofluorescence corresponded exactly with the organization of adhesion plaques on the ventral cell surface. This association was confirmed by demonstrating that pp60^{src} still could be detected in substrate-bound adhesion plaques after removal of the main cell body. Furthermore, the temperature-sensitive pp60^{src} synthesized in tsNY68-infected CEF was found within adhesion

plaques only when the cells were grown at permissive temperature. The concordance between the activity of pp60^{src} at permissive temperature (4–6, 9, 15) and the localization within adhesion plaques suggests that these sites somehow may be involved in maintaining the transformed phenotype.

Studies using interference-reflection microscopy have indicated that cells in culture exhibit different regions of separation from the substratum on which they grow (19, 21). The adhesion plaques, sometimes called focal contact areas, represent specialized cellular membrane regions of nearest approach to the substratum (10–15 nm) and are discrete sites of cell–substratum adhesion. Other broader and more uniform areas of separation (approximately 30 nm), called close contact areas, may also play a role in adhesion. The remainder of the ventral cell membrane is separated by a greater distance (100–140 nm) and probably does not participate in adhesion. Of the two regions that mediate cell–substratum adhesion, the adhesion plaques are perhaps more significant (19, 21) and were the only sites on the ventral surface where pp60^{src} was detectable. This suggests that pp60^{src} at these sites could influence cell–substratum adhesion. Such an effect has been reported for transformed cells (25, 26); however, it is not entirely clear whether the adhesion assays measured the actual cell–substratum adhesion through adhesion plaque sites or whether these assays simply measured the ability to separate the cell from the substratum-bound adhesion plaque. The effect of pp60^{src} on this aspect of adhesion plaque function will require further study.

The adhesion plaques, while serving as static contact points to the substratum, also function in more dynamic aspects of cellular activity. In locomotion, the adhesion plaques have been observed to remain stationary with respect to the substratum. As the cell moves forward new adhesion plaques form close to the leading edge of the lamellapodium, while more posterior adhesion plaques at the trailing edge of the cell dissolve (20). On the cytoplasmic side of the ventral cell membrane, adhesion plaques have been found associated with the distal ends of microfilament bundles or "stress fibers" (19–21, 27). The adhesion plaques therefore serve as components of the cytoskeleton that anchor the fibrillar network to the substratum. In the advancing lamellae, adhesion plaque placement occurs synchronously with microfilament bundle formation (21). The microfilament bundles generally extend from the adhesion plaques under the nucleus toward the posterior of the cell (28, 29). Fibronectin has been postulated to participate in the formation of attachment plaques through transmembrane connections with microfilaments (30). This organization of microfilaments into bundles and their transmembrane anchorage at the adhesion plaque sites are believed necessary for cellular locomotion. The influence of pp60^{src} on cell motility is not adequately understood; however, it is difficult to imagine that the presence of pp60^{src} within adhesion plaques would not influence at least some aspects of cell locomotion.

In the tsNY68-infected cells, adhesion plaque formation occurred at both permissive and nonpermissive temperature, but only the adhesion plaques of cells maintained at permissive temperature contained pp60^{src}. Some morphological differences were apparent between adhesion plaques of normal and RSV-tumor cells; however, the above results suggest that pp60^{src} does not influence the formation of these structures but may affect their subsequent function. What that exact function may be is currently unknown, but, perhaps, it could be related to the disorganization of microfilament bundles.

Finally, these results provide evidence that pp60^{src} can be found associated with the adhesion plaque components of the cytoskeleton. These adhesion plaques serve as important bridges connecting the internal cytoskeleton with the external tactile environment. Therefore the presence of pp60^{src} at these sites may have a profound and coordinate influence not only on internal and external functions but perhaps also on the ability of the cell to respond to the external environment.

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