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Ultrastructural localization of a platelet-derived growth factor/v-sis-related protein(s) in cytoplasm and nucleus of simian sarcoma virus-transformed cells

(growth factors/oncogenes)

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ABSTRACT **The subcellular distribution of v-sis-related protein(s) was analyzed in three simian sarcoma virus (SSV)-transformed cell lines with immunofluorescence and protein A–gold labeling techniques using rabbit polyclonal anti-platelet-derived-growth factor (PDGF) antisera. Antigenically reactive proteins were recognized in subcellular organelles related to protein synthesis and processing, including polyribosomes, endoplasmic reticulum, and the Golgi apparatus, as well as on the cytoplasmic surface of plasma membranes. Prominent immunoreactive proteins were also shown in association with nuclear chromatin in intact cells and in isolated nuclei using Lowicryl K_4M resin embedding techniques. Protein A–gold labeling was markedly reduced in sections of non-SSV-transformed fibroblasts incubated with anti-PDGF and absent from SSV-transformed cells if Epon resin was substituted for Lowicryl in the embedding process or if sections were with irrelevant antisera. Nuclear localization of v-sis-related antigens was confirmed in a nitrocellulose-based immunoassay using nuclei isolated from SSV-transformed fibroblasts. Thus, polypeptides recognized antigenically as related to the v-sis gene product not only may be found in subcellular organelles associated with protein synthesis and packaging but also may be found in the nucleus of SSV-transformed cells. These results raise the possibility that v-sis- or PDGF-like proteins may function within the nucleus of SSV-transformed cells.**

The v-sis gene product ($p28^{v\text{-}sis}$) of the simian sarcoma virus (SSV) structurally and functionally is highly homologous to platelet-derived growth factor (PDGF) (1–4). The protein is expressed in SSV-transformed cells and a PDGF-like protein is secreted into conditioned medium that binds to the PDGF cell-surface receptor and that appears to stimulate the autocrine growth of SSV-transformed cells (5, 6). The processing of $p28^{v\text{-}sis}$ has been investigated using subcellular fractionation and immunoprecipitation of labeled SSV-transformed cell extracts with antisera generated against terminal peptide fragments of sis (7), but detailed ultrastructural localization studies are not available. Subcellular localization of viral and cellular oncogenes has been used to suggest functions of these proteins and to suggest sites at which transformation may be initiated (8, 9). Light and/or electron microscopic immunocytochemistry has effectively localized several oncogene products, such as $p60^{src}$, $p21^{ras}$, and the v-myc and c-myc proteins (10–13). The roles of proteins encoded by the *src* or *ras* oncogenes, which are found in the cytoplasm, plasma membrane, or both, may be quite different from the protein products of the *myc*, *myb*, and *fos* genes, which are localized in nuclei (10, 11, 14).

We used immunolabeling of three SSV-transformed cell lines with two polyclonal anti-PDGF antisera and protein A–colloidal gold (pA–Au) to provide additional information on potential sites of v-*sis* gene expression and activity. Electron micrographs of labeled cells demonstrated that antigens recognized by anti-PDGF antisera were located not only in ultrastructures related to protein synthesis and processing but also were prominently associated with nuclear chromatin. Nontransformed cells had markedly diminished amounts of immunoreactive protein. The functional activity of the PDGF/v-sis-related antigen(s) localized in the nucleus and whether it has a role in transformation by SSV are not known, but the results raise the possibility that v-sis-like polypeptides such as $p28^{v\text{-}sis}$ or its processed products may function at the level of the nucleus, perhaps in association with the PDGF receptor.

MATERIALS AND METHODS

Cells and Cell Culture. Three SSV-transformed cell lines [SSV-transformed marmoset fibroblasts, SSV-NP1 cells; SSV-transformed normal rat kidney (NRK) cells, SSV-NRK cells; SSV-transformed NIH 3T3 cells, SSV-3T3 cells] were grown as subconfluent or confluent monolayers in Dulbecco's modified Eagle's medium (DME medium) containing 10% fetal calf serum and antibiotics (100 units of penicillin per ml, 100 μg of streptomycin per ml) in 35-mm tissue culture dishes or in 75-cm^2 flasks for immunofluorescent or electron microscopic immunocytochemical analysis, respectively, as described (6). Nontransformed NRK cells and NIH 3T3 cells were used as normal controls. Cells were cultured with plasma-derived serum to replace fetal calf serum in medium 6–12 hr before fixation.

Antibody and Antibody Controls. Purified anti-PDGF IgG was prepared from anti-human PDGF antiserum raised in a New Zealand White rabbit as described (15). A second anti-PDGF antiserum was used; it was raised in a Flemish rabbit using highly purified PDGF (16) as antigen, processed with protein A affinity chromatography, and further purified on a PDGF-Sepharose affinity column. Affinity-purified fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Boehringer Mannheim) was used as second antibody for immunofluorescent studies. Control reagents used in establishing specificity of immunolabeling included (*i*) anti-PDGF IgG preadsorbed with excess PDGF for 1 hr at 37°C and for 72 hr at 4°C, (*ii*) "flow through," IgG passed over the PDGF affinity column four times and shown to have minimal anti-PDGF activity by solid-phase immunoassay (data not shown), (*iii*) nonimmune serum, (*iv*) anti-canavalia ensiformis

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Abbreviations: SSV, simian sarcoma virus; pA–Au, protein A–colloidal gold; PDGF, platelet-derived growth factor; FITC, fluorescein isothiocyanate; NRK, normal rat kidney.

lectin IgG (Sigma), 1:100 diluted in phosphate-buffered saline (PBS) at pH 7.2 substituted for anti-PDGF IgG, and (*v*) FITC-IgG or pA–Au (15 nM, Jansson Life Science Products, Piscataway, NJ), 1:10 diluted alone.

Indirect Immunofluorescent Method. Cells were grown as subconfluent or confluent monolayer cultures in 35-mm tissue culture dishes and were fixed with cold 95% ethanol (EtOH) for 5 min. Other fixatives were used but were found to be associated with loss of antigenicity to anti-PDGF antisera. Anti-PDGF IgG or control solutions (described above) were applied between the cover slide and the dish, incubated for 45 min at 37°C in a humidified chamber, and washed three times with PBS (pH 7.2), and the FITC-conjugated goat anti-rabbit IgG was added and incubated for 45 min at 37°C. After washing in PBS, the cells were mounted in 50% glycerol. Photographs were taken at identical exposure times using a Nikon microscope equipped with epifluorescent optics and a 40× objective (phase contrast), using Kodak Tri-X (ASA 400) film.

Ultrastructural Immunocytochemistry. *Sample fixation.* Cells were grown in 75-cm^2 flasks. After three washes with 0.2 M sodium cacodylate buffer (pH 7.2), the cells were fixed *in situ* with 1% glutaraldehyde in 0.1 M sodium cacodylate buffer for 30 min at room temperature. They were then washed three times with 0.2 M sodium cacodylate buffer and treated with 0.1 M NH_4Cl for 15 min to neutralize glutaraldehyde. The cells were removed with a rubber policeman, centrifuged at 1500 rpm for 10 min, and pelleted in 1% agar.

Isolated nuclei were prepared by using a low-salt, nonionic detergent method after cells were treated with trypsin, using a modification of the method of Armbruster *et al.* (17). The treated cells were washed twice with 0.15 M sucrose in 0.2 mM phosphate buffer (pH 7.5) and lysed by exposure for 5 min in 1:1 or 1:2 lysis buffer [0.5% Nonidet P-40 (vol/vol), 0.2 mM EDTA, and 0.2 mM phenylmethylsulfonyl fluoride, pH 7.5]. The lysate was washed twice with 0.15 M sucrose in 0.2 mM phosphate buffer and centrifuged at 2500 rpm for 7 min. Purity and integrity of the nuclei preparations were monitored by microscopy under phase optics. The precipitate was fixed immediately in 1% glutaraldehyde/0.1 M sodium cacodylate and pelleted into 1% agar. Prior to the solid-phase immunoassay, the isolated and washed nuclei were stored at −20°C until use.

Dehydration and embedding. The agar pellets were dehydrated by exposure successively to increased concentrations of EtOH at progressively lower temperatures—specifically, specimens were processed in 30% EtOH (0°C, 30 min), 50% EtOH (−20°C, 60 min), 70% and 95% EtOH (−35°C, 60 min, respectively), and 100% EtOH (two times, −35°C, 60 min); this was followed by infiltration with Lowicryl K_4M fixative at −35°C in a mixture of 100% EtOH, 1:1 (60 min), 2:1 (4 hr), and pure K_4M (overnight). Pellets were placed on glass slides in pure K_4M and polymerized by long-wavelength (360 nm) indirect UV light at −35°C for 24 hr and then cured at room temperature for 48 hr.

Immunocytochemical methods. Thin sections on nickel grids were incubated with 1% bovine serum albumin in PBS (pH 7.2) for 30 min to block nonspecific attachment of antibodies to residual glutaraldehyde. After "jet" washing with PBS for 60 sec, samples were allowed to react with anti-PDGF IgG or control solutions for 90 min. After jet washing again with PBS (60 sec per grid), the grids were incubated with pA–Au complexes for 40 min and then washed extensively with PBS. The sections were doubly stained with saturated aqueous uranyl acetate in the presence or absence of lead citrate and viewed with Phillips 210c electron microscope

Solid-Phase Immunoadsorbant Assay. A modification of the procedure developed by Hawkes *et al.* (18) was used. Nitrocellulose (BA85, 0.45 μm, Schleicher & Schuell) was immersed briefly in water, then applied to the Minifold II (Schleicher & Schuell), and allowed to dry. Samples containing antigen were applied to the slots for 60 min; remaining sites were blocked with 5% bovine serum albumin (Sigma) for 30 min. First antibody (anti-PDGF) was added at appropriate dilutions for 60 min, and the sheets were washed three times in 50 mM Tris·HCl/200 mM NaCl, pH 7.4, containing 0.3% (vol/vol) Tween 20 (Sigma). Peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad) diluted to 1:3000 was added for 1 hr and the wash procedure was repeated. The chromogen, 4-chloro-1-naphthol (Bio-Rad), 3 mg/ml in methanol, was diluted 1:5 in buffer containing hydrogen peroxide (0.03%, vol/vol) and applied to the samples for 20 min. Color development was stopped with water, and the stained nitrocellulose was photographed for analysis.

RESULTS

Localization of v-sis-Like Antigens by Immunofluorescent Microscopy. Three SSV-transformed and two nontransformed cell lines were examined by indirect immunofluorescence. After incubation with anti-PDGF IgG and FITC-conjugated goat anti-rabbit IgG, bright green fluorescence was detected within transformed cells. Fluorescently labeled cells were rarely detected in nontransformed cultures. The fluorescence intensity was variable within the transformed cell population and was dependent on cell density (Fig. 1), as assessed by phase-contrast microscopy. Fluorescence was diffuse throughout cytoplasm in SSV-NP1 cells and SSV-3T3 cells but was localized more intensely on the cytoplasmic surface areas and within some nuclei of subconfluent cultures

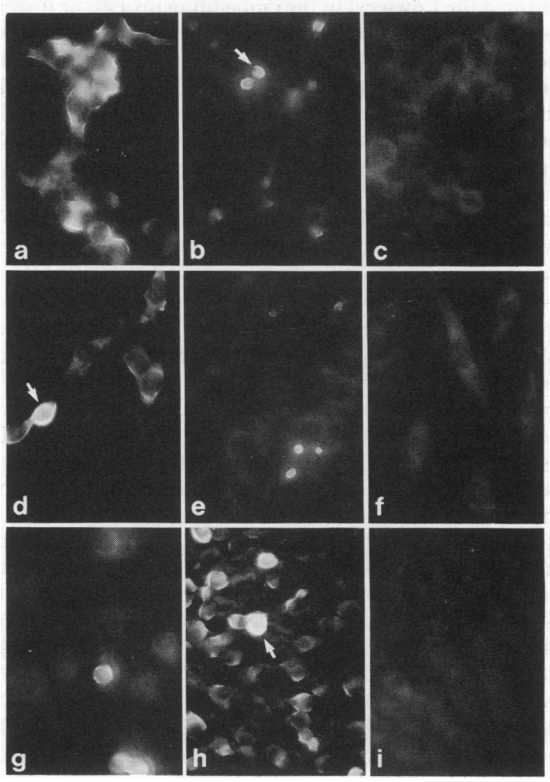

FIG. 1. Localization of p28$^{v\text{-}sis}$-like proteins in SSV-transformed cells by the indirect immunofluorescent method. Subconfluent cultures and confluent cultures of SSV-NP1 cells (*a* and *b*), SSV-3T3 cells (*d* and *e*), and SSV-NRK cells (*g* and *h*), respectively, show variable intensity of fluorescence in the cytoplasm, cytoplasmic surface of the plasma membrane, and nuclei (arrowheads in *b*, *d*, and *h*). SSV-NP1 cells that reacted with PDGF-adsorbed anti-PDGF IgG instead of anti-PDGF IgG (*c*), NIH 3T3 cells (*f*), and NRK cells (*i*) are controls. (×400.)

(Fig. 1 *a* and *d*); fluorescence was observed in far fewer confluent cells (Fig. 1 *b* and *e*). In contrast to results with SSV-NP1 and SSV-3T3 cells, SSV-NRK cells showed greater fluorescence in confluent cultures (Fig. 1*h*) than in subconfluent cultures (Fig. 1*g*). Cells with positive fluorescence were generally round in shape and had overgrown one another at high density. In these cells, fluorescence was distinctly localized in the nuclei and was more intense in the nuclear margins (Fig. 1*h*). In control experiments, nontransformed NRK cells and NIH 3T3 cells were incubated with anti-PDGF IgG and fluorescent anti-rabbit IgG antisera. Significant fluorescence above background was rarely observed (Fig. 1 *f* and *i*). Furthermore, when three SSV-transformed cells were analyzed for immunofluorescence after incubation with anti-PDGF IgG pretreated with PDGF, with antilectin IgG, or with PBS alone, instead of with anti-PDGF IgG, fluorescent staining was not observed over background levels (Fig. 1*c*).

Ultrastructural Localization of v-sis-Like Antigens. The ultrastructure of the SSV-transformed and nontransformed cells embedded at low temperature in Lowicryl K_4M resin was found to be well preserved. The nuclei of the transformed cells were large and contained chromatin in excess of normal cells, as evident in ultrathin sections (data not shown). After exposure to anti-PDGF IgG and pA–Au, pA–Au particles were clearly identified associated with the ultrastructure of transformed cells (see Figs. 2–5). Background labeling in these sections was low. In Fig. 2, in the absence of lead citrate staining, pA–Au particles were primarily localized to the nucleus; however, staining was also observed in the cytoplasm and in microvilli. pA–Au was typically observed in higher density over the peripheral regions of the nucleus, which are usually abundant in heterochromatin (Fig. 2). Staining was also concentrated over the outer parts of the cytoplasm and microvilli in many of the transformed cells (Fig. 2 *Inset*). The labeling in the electron micrographs thus corresponds precisely to the immunofluorescent patterns demonstrated in Fig. 1. In the cytoplasm, pA–Au particles were observed in the membranes of the endoplasmic reticulum and were associated with ribosomes. To a lesser but significant extent, immunoreactive protein was found within the lumin of the endoplasmic reticulum (Fig. 3). Quantitatively less pA–Au labeling was observed in the Golgi apparatus; in a developed Golgi apparatus, only a few pA–Au particles were present in the cisternae and small vesicular membranes (Fig. 4). Nevertheless, particles were consistently associated with the Golgi in repeated experiments. The density of pA–Au particles localized to specific ultrastructural organelles was variable from cell to cell within each of the three SSV-transformed cell lines studied, perhaps reflecting differences in the cell cycle among the cells studied. Similar immunocytochemical analyses of control, SSV-NRK cells that had reacted with nonimmune serum and non-SSV-transformed NRK and NIH 3T3 cells were done by using the same concentations of anti-PDGF IgG and pA–Au as were used with SSV-transformed cells. Only low levels of labeling with pA–Au were observed (Fig. 4 *Insets a* and *b*, respectively) and did not appear to be associated with specific subcellular organelles. Preadsorbed anti-PDGF IgG, flow through IgG from a PDGF affinity column, antilectin IgG, and PBS substituted for anti-PDGF were used in parallel with the labeling procedures above, and again only very low amounts of nonspecific background binding of pA–Au were found (data not shown).

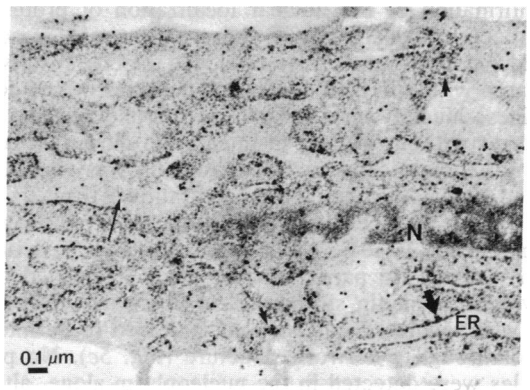

FIG. 3. Specific pA–Au labeling for $p28^{v\text{-}sis}$-like proteins in membrane ribosomes of endoplasmic reticulum (ER, thick arrow), free ribosomes, and polysomes (arrowhead) in SSV-3T3 cells. Only a few pA–Au particles are observed in the luminae of endoplasmic reticulum (thin arrow). (×19,500.)

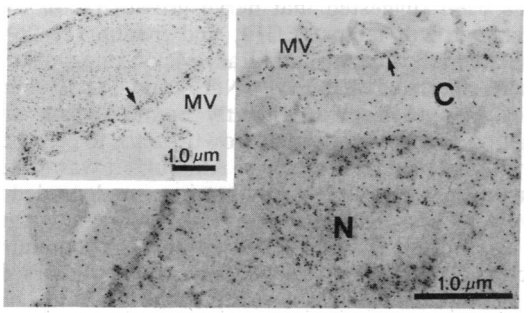

FIG. 2. Immunolocalization of $p28^{v\text{-}sis}$-like proteins in a thin section of an SSV-NP1 cell by the pA–Au technique (no lead citrate staining): labeling over nucleus (N), cytoplasm (C), outer parts of the cytoplasm (arrowhead), and microvilli (MV). (×11,000.) (*Inset*) Part of the cytoplasm of another SSV-NP1 cell. Increased pA–Au particles are present near the cytoplasmic surface of the plasma membrane (arrowhead) and microvilli (MV). (×5000.)

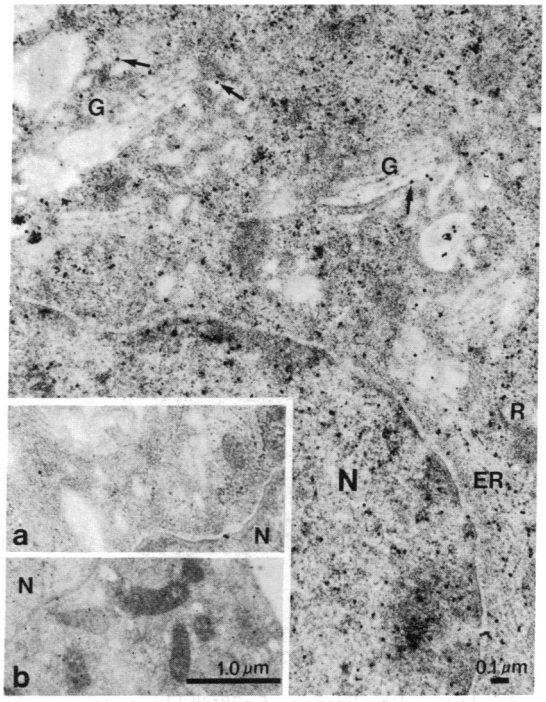

FIG. 4. Immunolocalization of $p28^{v\text{-}sis}$-like proteins in a thin section of an SSV-NRK cell. Only a few pA–Au particles (arrow) label the developed Golgi apparatus (G), but labeling is greater in the nucleus (N), endoplasmic reticulum (ER), ribosomes (R), and cytosol. (×19,000.) (*Inset*) Very low amounts of pA–Au particles in a part of SSV-NRK cell that reacted with nonimmune serum (*a*, ×19,000) and NRK cell that reacted with anti-PDGF IgG (*b*, ×12,000.)

Confirmation of the nuclear localization of protein immunoreactive with anti-PDGF antisera was sought in isolated nuclei from SSV-transformed cells, and then the results were compared with nuclei isolated from nontransformed control cell lines. Nuclei were isolated as morphologically intact structures without evident disintegration during processing (data not shown). The nuclear chromatin was variably distributed, depending upon the concentration of the lysis buffer used. pA–Au particles were specifically observed on nuclear chromatin of isolated SSV-NP1 cell nuclei (Fig. 5). In isolated nuclei, the pA–Au particles were easily delineated on the unraveled chromatin strands or on fibers that individually were well separated out but that appeared to be interconnected through a network structure (Fig. 5*c*). No pA–Au particles were detected in the nucleoplasm alone, although much of the nucleoplasm may have been removed in preparation. Thus, the antigenic material demonstrated within the nucleus in sections of whole cells was clearly associated with nuclear chromatin in isolated nuclei of SSV-transformed cells. Anti-PDGF antisera as well as anti-PDGF purified over a PDGF affinity column gave identical staining patterns.

The isolated nuclei were further analyzed in a nitrocellulose-based solid-phase immunoassay. Two anti-PDGF antisera were used with a peroxidase-conjugated second antibody for detection of the chromogen. Immunoreactive protein was clearly and specifically identified in nuclei of SSV-transformed cells (Fig. 6).

DISCUSSON

The amino acid sequence of the sis chain of PDGF (1, 2) has striking homology to the predicted amino acid sequence of $p28^{v\text{-}sis}$ (19), the transforming protein of the SSV. Anti-PDGF antisera have been used to establish the presence of a PDGF-like growth-promoting activity, presumed to be

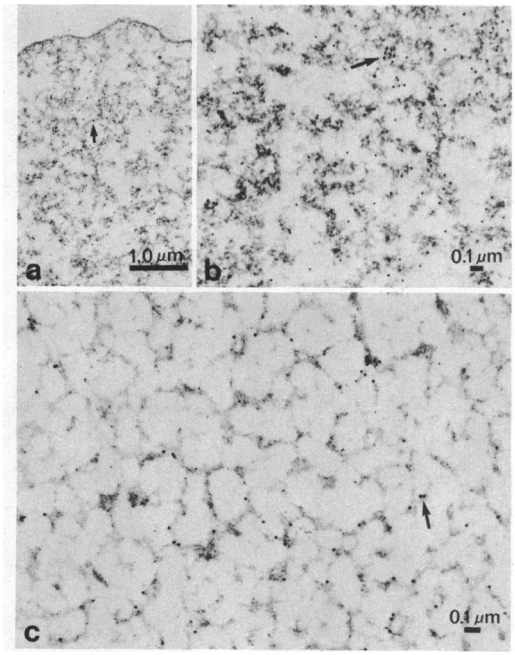

FIG. 5. Immunolocalization of $p28^{v\text{-}sis}$-like proteins in thin sections of an isolated nucleus of SSV-NP1 cell. pA–Au particles are seen in differentially spread chromatin. (*a*) pA–Au particles (arrow) are present in higher concentrations in chromatin over background nucleoplasm. (×9150.) (*b*) Part of *a*. (×16,900.) (*c*) pA–Au immunolabeling (arrow) is observed in chromatin strands or fibers that appear to be interconnected forming a network when isolated as described (17). No pA–Au particles are detected in the nucleoplasm. (×18,200.)

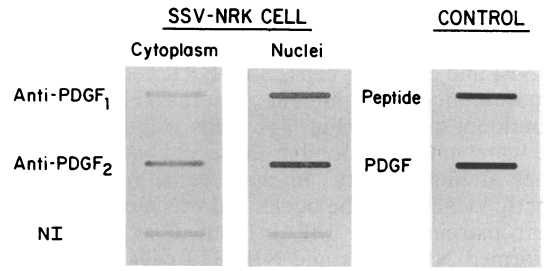

FIG. 6. Reactivity of SSV-NRK nuclei with two PDGF/v-sis-specific antisera in a solid-phase ELISA. SSV-NRK cytoplasm and nuclei (7.5×10^5 cell equivalents per slot) were assayed. Controls consisted of A chain peptide (1 μg) that reacted with anti-$PDGF_1$ IgG, PDGF (25 ng) that reacted with anti-$PDGF_2$, and nonimmune (NI) IgG. Other controls that used antisera or antigens alone were negative (data not shown).

$p28^{v\text{-}sis}$ or its processed product, in SSV-transformed cells (3) and in conditioned media from some but not all SSV-transformed cells (6). The *in situ* localization of these proteins in SSV-transformed cells has now been studied by using immunofluorescence and electron microscopic immunocytochemical methods to suggest sites of processing and potential roles in maintaining the transformed phenotype.

Immunolabeling with pA–Au combined with low-temperature embedding procedures using Lowicryl K_4M (17, 20) effectively preserved antigenicity and permitted the *in situ* detection of v-sis-like antigens with precision. Proteins antigenically similar to PDGF and the v-sis gene product were thus identified in patches of nuclear chromatin in thin sections of intact SSV-transformed cells or in association with network-like chromatin strands in isolated nuclei. When Epon resin-embedded SSV-transformed cells (data not shown) were sectioned, the colloidal gold method for detecting immunoreactive proteins failed to detect antigenic v-sis-like or PDGF-like proteins.

Robbins *et al.* (7) localized $p28^{v\text{-}sis}$ and its processed products in the endoplasmic reticulum, in the Golgi apparatus, and in the submembrane fractions of SSV-transformed cells using immunoprecipitation of labeled subcellular fractions. The most stable processed product of $p28^{v\text{-}sis}$, $p24^{v\text{-}sis}$, is thought to function as an autocrine stimulator of cell growth, probably by way of interaction with the PDGF receptor upon secretion (6, 21). The present experiments demonstrate that a protein(s) antigenically related to or identical with the product of the v-*sis* gene is located in the nucleus of SSV-transformed cells in addition to its association with organelles associated with protein processing, raising the possibility that $p28^{v\text{-}sis}$, its processed product, or related gene products may regulate nuclear events as well.

It has been suggested that nerve growth factor (NGF), epidermal growth factor (EGF), and insulin (22–26) may accumulate in the nucleus, and Rakowicz-Szulczynska *et al.* (27) have suggested that EGF, NGF, and PDGF could be detected in cytoplasm and tightly bound to the nuclear chromatin 1 hr after addition of growth factor to cells bearing the appropriate surface receptor. Although it was suggested that the nuclear growth factors were in a nondegraded form, data were not presented to support the idea that intact growth factor was localized to the nucleus nor was the contamination of nuclear fractions by cytoplasmic growth factors during isolation excluded. Our results exclude the artifactual binding of v-sis-like proteins to the nucleus but leave open the precise identity of the nuclear v-sis-like protein. How this protein is transported to the nucleus is likewise not resolved. PDGF and $p28^{v\text{-}sis}$ contain a sequence, Arg-Lys-Lys-Pro-Ile-Phe-Lys-Lys-Ala-Thr, from residues 190–199, that is homologous to the nuclear recognition site on the large tumor antigen (28, 29), suggesting one possible mechanism for nuclear trans-

port. Curiously, v-sis mutagenized by deletion was observed to localize in the nucleus, whereas intact v-sis protein was not (29). Since the v-*sis* mutation was nontransforming, the biologic implications of nuclear localization in this system are also unknown. Whether or not the PDGF receptor is required for migration of the v-sis-like protein to the nucleus has not yet been investigated.

Nuclear oncogene products may be found with DNA and RNA tumor viruses (30). Several of these have structural homology with one another (31–33). The roles of oncogene products localized in the nucleus remain to be established, although a role as trans-acting transcriptional regulators of specific genes relating to cell division seems likely. The present investigation does not establish a functional activity for the nuclear antigen(s) recognized by the different anti-PDGF antisera; $p28^{v\text{-}sis}$ is a highly basic protein and thus potentially may bind to nuclear chromatin through its very strong net positive charge at physiological pH, while limiting its biologic function to extranuclear sites. The antigen recognized by the anti-PDGF antisera is likely a closely related gene product to $p28^{v\text{-}sis}$; its increased expression is associated with transformation by the v-sis gene product. A second candidate protein product might be the homodimeric gene product of the non-c-*sis*-encoding gene of PDGF (A chain); this chain is recognized by the anti-PDGF antisera used in these experiments.

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1. Waterfield, M. D., Scrace, G. T., Whittle, N., Stroobant, P., Johnsson, A., Wasterson, A., Westermark, B., Heldin, C.-H., Huang, J. S. & Deuel, T. F. (1983) *Nature (London)* **304,** 35–39.
2. Doolittle, R. F., Hunkapiller, M. W., Hood, L. E., Devare, S. G., Robbins, K. C., Aaronson, S. A. & Antoniades, H. N. (1983) *Science* **221,** 275–277.
3. Deuel, T. F., Huang, J. S., Huang, S. S., Stroobant, P. & Waterfield, M. D. (1983) *Science* **221,** 1348–1350.
4. Robbins, K. C., Antoniades, H. N., Devare, S. G., Hunkapiller, M. W. & Aaronson, S. A. (1983) *Nature (London)* **305,** 605–608.
5. Garrett, J. S., Coughlin, S. R., Niman, H. L., Tremble, P. M., Giels, G. M. & Williams, L. T. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 1193–1197.
6. Huang, J. S., Huang, S. S. & Deuel, T. F. (1984) *Cell* **39,** 79–87.
7. Robbins, K. C., Leal, F., Pierce, J. H. & Aaronson, S. A. (1985) *EMBO J.* **4,** 1783–1792.
8. Land, H., Parada, L. F. & Weinberg, R. A. (1983) *Science* **222,** 771–778.
9. Bishop, J. M. (1985) *Cell* **42,** 23–38.
10. Hann, S. R., Abrams, H. D., Rohrschneider, L. R. & Eisenman, N. (1983) *Cell* **34,** 784–798.
11. Alitalo, K., Ramsay, G., Bishop, J. M., Pfeifer, S. O., Colby, W. W. & Levinson, A. D. (1983) *Nature (London)* **306,** 274–277.
12. Willingham, M. C., Jay, G. & Paston, I. (1979) *Cell* **18,** 125–134.
13. Willingham, M. C., Pastan, I., Shih, T. Y. & Scolnick, E. M. (1980) *Cell* **19,** 1005–1014.
14. Rohrschneider, L. R. & Gentry, L. E. (1984) in *Mechanisms of Neoplastic Transformation at the Cellular Level,* Advances in Viral Oncology, ed. Klein, G. (Raven, New York), Vol. 4, pp. 269–306.
15. Huang, J. S., Huang, S. S. & Deuel, T. F. (1983) *J. Cell Biol.* **97,** 383–388.
16. Deuel, T. F., Huang, J. S., Proffitt, R. T., Baenziger, J. U., Chang, D. & Kennedy, B. B. (1981) *J. Biol. Chem.* **256,** 8896–8899.
17. Armbruster, B. L., Garavito, R. M. & Kellenberger, E. (1983) *J. Histochem. Cytochem.* **31,** 1380–1384.
18. Hawkes, R., Niday, E. & Gordon, J. (1982) *Anal. Biochem.* **119,** 142–147.
19. Devare, S. G., Reddy, P., Law, J. D., Robbins, K. & Aaronson, S. A. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 731–735.
20. Roth, J. (1982) in *Techniques in Immunocytochemistry,* eds. Bullock, G. R. & Petruz, P. (Academic, London), Vol. 1, pp. 108–133.
21. Johnsson, A., Betsholtz, C., Heldin, C.-H. & Westermark, B. (1986) *EMBO J.* **5,** 1535–1541.
22. Yanker, B. A. & Shooter, E. M. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 1269–1273.
23. Andres, R. Y. & Bradshaw, R. A. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 2785–2789.
24. Johnson, L. K., Vlodavsky, I., Baxter, J. D. & Gospodarowicz, D. (1980) *Nature (London)* **287,** 343–348.
25. Savion, N., Vlodavsky, I. & Gospodarowicz, D. (1981) *J. Biol. Chem.* **256,** 1149–1154.
26. Goldfine, I. D., Smith, G. J., Wong, K. Y. & Jones, A. L. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 1368–1372.
27. Rakowicz-Szulczynska, E. M., Rodeck, U., Herlyn, M. & Koprowski, H. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 3728–3732.
28. Smith, A. E., Kalderon, D., Roberts, B. L., Colledge, W. H., Edge, M., Gillett, P., Markham, A., Paucha, E. & Richardson, W. D. (1985) *Proc. R. Soc. London Ser. B* **226,** 43–58.
29. Hannink, M. & Donoghue, D. J. (1986) *Mol. Cell. Biol.* **6,** 1343–1348.
30. Weinberg, R. A. (1985) *Science* **230,** 770–776.
31. Ralston, R. & Bishop, J. M. (1984) *Nature (London)* **306,** 803–806.
32. Schwab, M., Alitalo, K., Klempnauer, K.-H., Bishop, J. M., Gilbert, F., Brodeur, G., Goldstein, M. & Trent, J. (1983) *Nature (London)* **305,** 245–248.
33. Kohl, N. E., Kanda, N., Schreck, R. R., Bruns, G., Latt, S. A., Gilbert, F. & Alt, F. W. (1983) *Cell* **35,** 359–367.