JOURNAL OF VIROLOGY, May 1984, p. 623–628
0022-538X/84/050623-06$02.00/0

Vol. 50, No. 2

Localization of the Simian Virus 40 Small t Antigen in the Nucleus and Cytoplasm of Monkey and Mouse Cells

MATTHEW ELLMAN, ILAN BIKEL, JAMES FIGGE, THOMAS ROBERTS, ROBERT SCHLOSSMAN, AND DAVID M. LIVINGSTON*

The Dana-Farber Cancer Institute and Departments of Medicine and Pathology, Harvard Medical School, Boston, Massachusetts 02115

Received 4 November 1983/Accepted 10 January 1984

Monkey and mouse cells producing simian virus 40 small t antigen in the absence of clearly detectable intact or truncated large T antigens were subjected to indirect immunofluorescence and biochemical cell compartment analyses. Results revealed specific immunofluorescence and small t polypeptide in both the nucleus and cytoplasm of these cells.

The early region of simian virus 40 (SV40) encodes two known proteins, large T antigen (T) and small t antigen (t). Both of these elements are active in the process of virus-induced neoplastic transformation, although the mechanisms by which they operate in this regard are wholly unknown. Unlike T, it appears that t is dispensable during productive infection (14), although under some conditions both proteins play essential, albeit complementary, roles in the transforming process (4, 7, 13, 16, 18). T can be readily identified in infected cells by a variety of techniques, including indirect immunofluorescence, which shows it to be largely a nuclear constituent. By contrast, it has not been possible to detect t immunofluorescence in the past. The cellular location of this protein could, therefore, be assessed only by biochemical extraction studies which suggested that it was largely a constituent of the postnuclear soluble fraction and might, therefore, be largely, if not wholly, a cytoplasmic constituent (10, 17). Comparable results were obtained for polyomavirus (15). However, in view of the indirect nature of the analytical approach employed in all of these studies, a detailed understanding of the intracellular geography of t was not possible. In an effort to address this problem, we have raised an antibody against homogeneous SV40 t synthesized in *Escherichia coli* (2, 12) and used it to decorate cells which synthesize substantial levels of t, but no readily detectable intact or truncated T by indirect immunofluorescence. Results of these experiments revealed that t fluorescence can be readily identified in both the nucleus and cytoplasm of two different cell types. As expected, in the same types of experiments, T fluorescence was noted to be largely, if not exclusively, nuclear.

We have recently described a plasmid (pHR402) and a derivative SV40 mutant virus (SV402), which, upon introduction into rodent or monkey cells, lead to the synthesis of t but not clearly or reproducibly detectable quantities of T or a truncated derivative thereof (13). The 402 genome contains two tandemly oriented viral replication origins upstream of an intact t-coding unit. The 402 early region has also sustained a ≈1.5-kilobase deletion of T-unique coding sequences.

When pHR402 was cotransfected onto NIH-3T3 cells in the presence of pSV2 *Ecogpt* (8), some of the clones which grew in medium containing mycophenolic acid also synthesized intact SV40 20-kilodalton t and failed to accumulate readily detectable quantities of intact or truncated T, as assayed by specific immunoprecipitation of [^{35}S]methionine-labeled cell extracts. One such example is shown in Fig. 1. The rabbit antiserum used in these experiments was raised against sodium dodecyl sulfate (SDS)-gel band-purified t synthesized in *E. coli* (2). This serum recognizes both t and T but failed to bind t after its adsorption to an extract of monkey cells infected by a T^+/t^- virus, *dl*884 (2, 14). Hence, the vast majority if not all of the relevant antibody molecules in this serum recognize an antigen(s) contained within the common T/t sequence. In an experiment analogous to that reported above, CV-1P cells were acutely infected with SV402, and extracts of these cells were exposed to anti-T/t immunoprecipitation (Fig. 1). No cytopathic effect or production of T was observed in such SV402-infected cultures, and in keeping with this observation, only t was clearly identified by specific immunoprecipitation. In this regard, others have shown that mouse LTK cells transformed by a recombinant genome, pVBtTK-1, which contains an analogously deleted SV40 early region, also failed to accumulate readily detectable truncated T with the sera employed in the relevant immunoprecipitation assays (11). As a test of the possibility that expression of the 402 genome might lead to a truncated T which comigrated with t in SDS-polyacrylamide gels, extracts of NIH-3T3 clone 5-4 (one of the above-noted t^+/T^- *gpt*$^+$ cotransformants) and Cos-1 cells (a T^+/t^+ monkey cell line) were immunoprecipitated with the above-noted serum. Aliquots of the eluted products were then reduced with dithiothreitol and alkylated with N-ethylmaleimide (3). As noted in Fig. 1, comigrating t bands were noted in these two extracts before and after reduction and alkylation, and in each case, the migration rate of t slowed to the same degree after chemical modification, in keeping with the original observation of Crawford and O'Farrell (3). By contrast, no major alteration in Cos-1 T migration was detected; furthermore, no new immunoreactive bands were seen after the above-noted treatment. Since the putative truncated T molecule encoded by the 402 early region (13) would lack t-unique coding sequences and be expected to have a different cysteine content than t (4 versus 11), the failure to detect a previously "hidden" 20-kilodalton band after reduction and alkylation is consistent with the notion that expression of the 402 genome does not lead to the intracellular accumulation of a truncated form of T which normally comigrates with t. Furthermore, a one-dimensional isoelectric focusing gel analysis of [^{35}S]-methionine-labeled pHR402 t from the NIH-3T3 *gpt*$^+$ cotransformant, clone 5-4, revealed that all of the specifically immunoprecipitated

* Corresponding author.

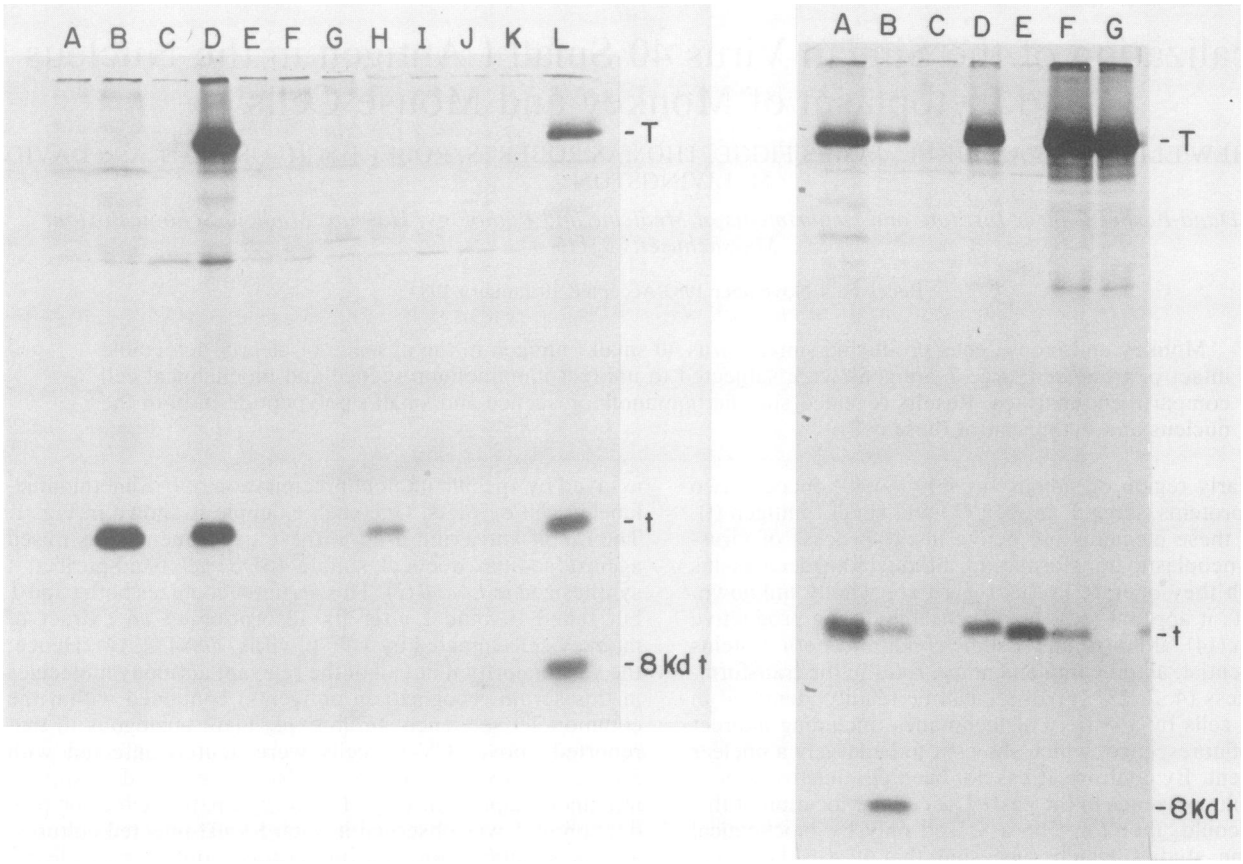

FIG. 1. Immunoprecipitation of [^{35}S]methionine-labeled SV40 T and t from various cell extracts. All cells were grown in Dulbecco modified Eagle minimal essential medium in a 10% CO_2-containing atmosphere. The conditions for virus production, virus titration, cell labeling with L-[^{35}S]methionine, cell lysis, the raising of SV40 t antibody against SDS-gel-purified protein, specific immunoprecipitation, SDS-polyacrylamide gel electrophoresis, and autoradiography have been described previously (2, 13, 17). SDS-gel electrophoresis was routinely performed in 15% polyacrylamide running gels cast beneath 4% stacking segments. Upper-left panel, Lanes B, D, F, H, J, and L: rabbit anti-t immunoprecipitates. Other lanes: preimmune rabbit serum precipitates. Extracts employed were as follows: SV402-infected CV-1P cells (lanes A and B); SV40 (strain 776, clone 1)-infected CV-1P cells (lanes C and D); CV-1P cells (lanes E and F); NIH-3T3 *Ecogpt*$^+$ cells (8) which were cotransformed with pHR402 (13) (clone 5-4) (lanes G and H); NIH-3T3 *Ecogpt*$^+$ clone 2-1 cells which lack SV40 sequences (lanes I and J); and SV80 cells (lanes K and L). SV80 synthesizes T, t, and an 8-kilodalton (kd) truncated T which is largely composed of SV40 T/t common sequences (17, 18). Upper-right panel, All lanes: rabbit anti-t serum precipitates of [^{35}S]methionine-labeled cell extracts. The following extracts were used: Cos-1 cells (5) (lane A), SV80 cells (lane B), uninfected CV-1P cells (lane C), SV40 (strain 776)-infected CV-1P cells (lane D), SV402-infected CV-1P cells (lane E), *dl*883-infected CV-1P cells (6, 14) (lane G), and *dl*883–SV402-infected CV-1P cells (lane F). The slight migration difference between SV40 (strain 776) t in lane D and the other t species here was specific for this viral clone. Bottom panel, Lanes A, B, E, and F: Cos-1 cell extract; lanes C, D, G, and H, SV402-infected CV-1P cell extract. Lanes A, C, E, and G, rabbit anti-t serum precipitates; lanes B, D, F, and H, preimmune rabbit serum precipitates. The SDS-eluted antigen(s) in lanes E through H had been exposed to 20 mM dithiothreitol and 40 mM *N*-ethylmaleimide before electrophoresis, as described previously (3). t_{ALK}, Migration position of reduced and alkylated t.

radioactivity which was detected appeared to comigrate with authentic SV40 t synthesized from an intact t-coding unit in *E. coli* (2, 12) (data not shown). Hence, by these criteria, all of the radioactivity in the anti-t-reactive 20-kilodalton band in cells bearing the 402 genome is SV40 t.

With the availability of a monospecific antibody which recognized one or more segments of the common T/t sequence and cells which, as characterized with this reagent, appear to accumulate t and not T, it was possible to search for this protein in fixed cells by indirect immunofluorescence. Thus, t^+/T^- *Ecogpt*$^+$-cotransformed NIH-3T3 and CV-1P cells acutely infected with SV402 were sequentially fixed in 3.7% formaldehyde followed by absolute methanol and then incubated with this antibody (Fig. 2 and 3). After a reaction with fluoresceinated goat anti-rabbit immunoglobulin G, distinct, bright fluorescence was noted in the nucleus and cytoplasm of both cell types. The intranuclear fluorescence was found to be diffuse and to spare evident nucleoli. Cytoplasmic fluorescence in many cells was noted to be "lacy" and, in part, to decorate a tight, albeit irregular, network of particulate material. Additional examples of these effects are noted in Fig. 3. No fluorescence was noted in NIH-3T3 cells transformed only with pSV2 *Ecogpt* (Fig. 2B), in uninfected CV-1P cells reacted with anti-t serum (Fig. 2E), or in SV40-infected CV-1P cells reacted with preimmune rabbit serum (data not shown). By contrast, CV-1P cells infected by the t^-/T^+ viral mutant *dl*883 (6, 14) contained only T (cf. Fig. 1, upper right panel, lane G) and revealed bright nuclear fluorescence without detectable cytoplasmic staining under the conditions employed (Fig. 2C). This result is in keeping with the widely appreciated observation that ≥90% of T in such cells is a nuclear constituent. Results qualitatively similar to those noted in the t-containing cells described here were obtained with the above-noted t^+/*Ecogpt*$^+$ NIH-3T3 clone 5-4 after fixation with methanol alone or with 3.7% formaldehyde followed by 1% Triton X-100 or acetone. In addition, identical results to those noted in Fig. 2 and 3 were obtained with the two other t^+/*Ecogpt*$^+$ NIH-3T3 clones (5-1 and 5-10) available in our laboratory. Hence, the nuclear and cytoplasmic staining effect is a reproducible finding in more than one cell type which synthesizes SV40 t in the absence of T. Moreover, similar, although less intense staining of fixed, 402 genome-containing t^+/T^- cells was obtained after incubation with one lot of pooled hamster anti-SV40 tumor serum. In keeping with the contention that the observed fluorescence is due to an immune reaction with t and not the predicted ≈20-kilodalton truncated species of T (which can, in theory, be encoded by the 402 genome), only t was detected by specific immunoprecipitation under the conditions employed with the same serum used in the indicated immunofluorescence experiments. Moreover, others have found that with hamster anti-SV40 tumor serum, T extending less than 272 amino acids from the normal N terminus failed to score in immunofluorescence assays (9).

In an independent test of the suggestion that t is present in both the nuclear and cytoplasmic cell compartments, clone 5-4 cells were fractionated into nuclei and a postnuclear soluble fraction after hypotonic swelling at 80 mM NaCl and Dounce homogenization performed by a modification of a previously described procedure (1). Nuclei were purified by differential centrifugation and washing. As determined by phase-contrast microscopy, the final pellet fraction was composed of ≥95% intact nuclei, and ≤2% intact cells were identified after examination of 100 intact nuclei. Purified nuclei were extracted in RIPA buffer (0.05 M Tris-hydrochloride [pH 7.4], 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS), and the resulting fractions were subjected to excess anti-SDS-gel band t immunoprecipitation. The results (Fig. 4) showed that t was present in both the nuclear and postnuclear soluble fractions. Densitometric tracing of the t gel bands revealed that ≈78% of the total immunoreactive t extracted from these two fractions was in the latter fraction, whereas ≈22% was in the nuclear fraction. No t was detected in the nuclear wash, and the finding of nuclear and soluble fraction-specific gel bands strongly suggested that little or no significant cross-contamination of these three fractions had occurred (Fig. 4, right panel). It should also be noted that when the same cells or SV402-infected CV-1P cells were swollen at 10 mM Na^+ before homogenization, ≤5% of the extracted t was present in the nuclear fraction (data not shown), suggesting that retention of t in the nucleus during and after cell disruption is, at least in part, a salt-sensitive phenomenon.

Thus, by two criteria, indirect immunofluorescence and biochemical extraction analysis, t appears to be distributed in two major cell compartments. The quantitative intracellular distribution between these locations is difficult to establish accurately, as the relative in situ immunoreactivities of nuclear and cytoplasmic t may not prove to be the same as they are in vitro. Moreover, it seems likely that some t leaks from the nucleus during hypotonic swelling or Dounce homogenization or both, and this may also have occurred during the experiment described in Fig. 4. In a similar experiment performed on SV40- or *dl*883-infected CV-1P cells, little or no T was detected in the postnuclear soluble fraction; nearly all of the antigen was present in the nuclear fraction (data not shown). Hence, the cell fractionation technique per se does not lead to indiscriminate loss of another known nuclear constituent.

In many cells, cytoplasmic t appeared to be distributed in a tightly packed, inhomogeneous pattern. This raises the possibility that it might, at least in part, be bound to one or more organized cell structures, e.g., a cytoskeletal component or a structure like the Golgi apparatus or endoplasmic reticulum. t has been shown to be able to bind to three cell proteins in vitro (19, C. Murphy, I. Bikel, and D. M. Livingston, submitted for publication). One of them is tubulin. Whether the others are associated with such structures is unknown.

The data presented here represent the first in situ demonstration of SV40 t and lead to the surprising conclusion that in addition to existing in the cytoplasm, t is also a nuclear component. Recently, a similar conclusion has been drawn from immunofluorescence experiments in other laboratories for polyomavirus (R. Kamen, personal communication) and BK virus (D. McCance and A. E. Smith, personal communication) t, two papovaviral proteins which are closely related to SV40 t. T is also present and functions in the nucleus, but since t was detected there in cells lacking T, its entry into that compartment is not a T-dependent process.

The significance of the dual distribution of t is unclear, but certain relevant possibilities can be cited. (i) Perhaps t is biologically active only in the nucleus and is transported there slowly or inefficiently, thereby creating a sizeable pool of nonfunctioning cytoplasmic t; (ii) t may exist largely in the nucleus and leak out into the cytoplasm during cell fixation or immunofluorescence manipulations or both; (iii) perhaps t functions in both the nucleus and the cytoplasm and, if so, in at least partially different ways in the two cell compartments; (iv) it is theoretically possible that t moves from the cytoplasm to the nucleus in the course of performing a discrete

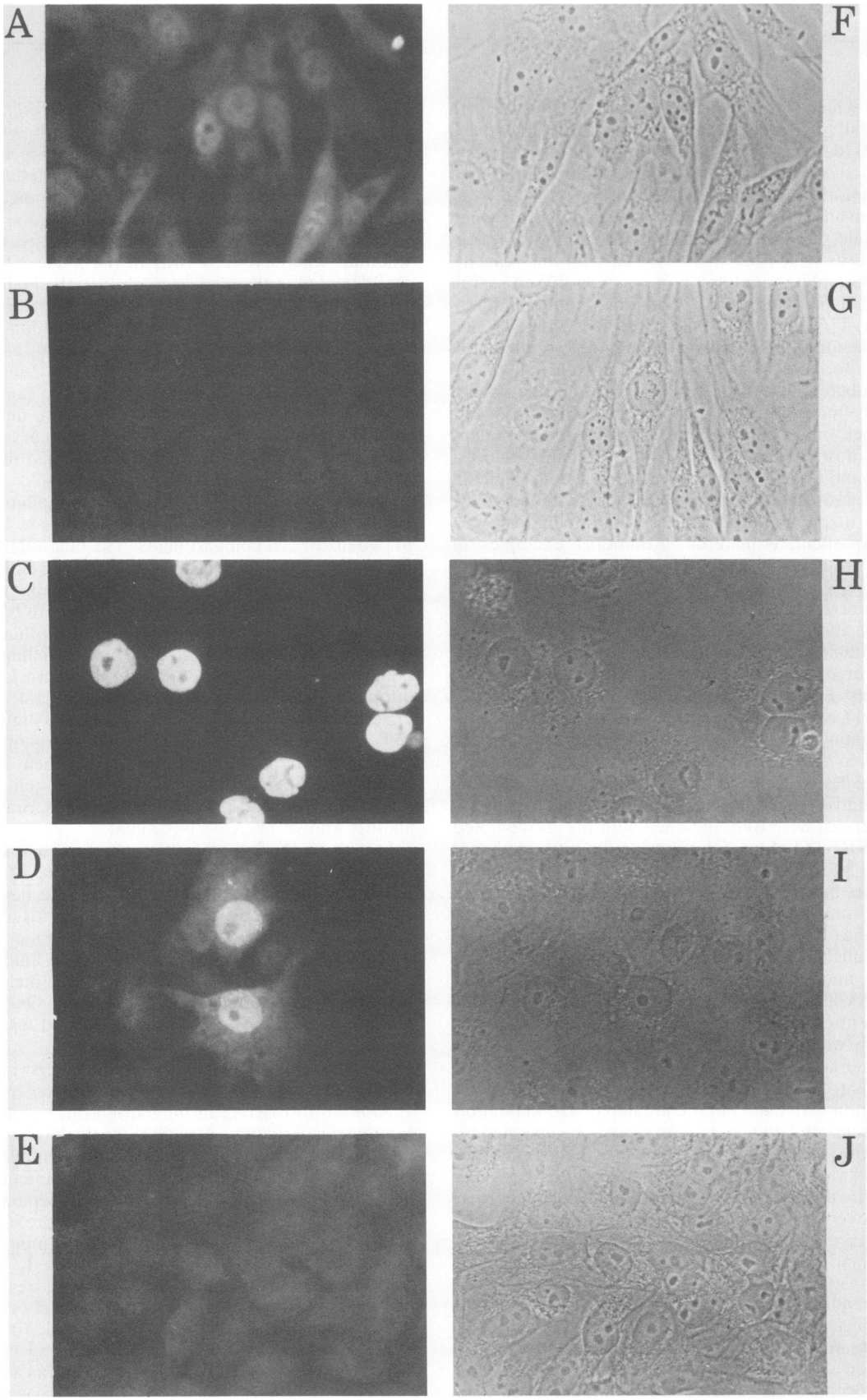

FIG. 2. Immunofluorescence of SV40 T and t in various cell lines. Cells were grown on glass cover slips, as noted in the legend to Fig. 1. They were then rinsed in phosphate-buffered saline (PBS) (0.01 M sodium phosphate [pH 7.4], 0.14 M NaCl) and immersed in 3.7% formaldehyde in PBS for 20 min at room temperature. Cover slips were again rinsed three times in PBS and then immersed in ice-cold absolute methanol for 2 min at −23°C. After three more rinses in room temperature PBS, the cover slips were drained of obvious surface fluid and then incubated with rabbit anti-SDS-gel band-purified t serum (1:40 dilution) for 30 min at 37°C in a humidified atmosphere. Subsequently, they were rinsed three times in PBS and then incubated with fluoresceinated goat anti-rabbit $F(ab')_2$ immunoglobulin G (1:100 dilution) (Cappel Laboratories), rinsed again three times in PBS, and then dried and mounted in 50% glycerol in PBS. Cells were viewed and photographed at ×40 magnification with a Zeiss inverted UV microscope under oil immersion. Left panels, Fluorescent images. Right panels, Bright-field, phase-contrast images. All panels show cells after incubation with the same rabbit anti-SDS-gel band-purified t serum described in the legend to Fig. 1. (A and F) NIH-3T3 *Ecogpt*$^+$/t$^+$ clone 5-4; (B and G) NIH-3T3 *Ecogpt*$^+$ clone 2-1, a cell line which was transfected only with pSV2

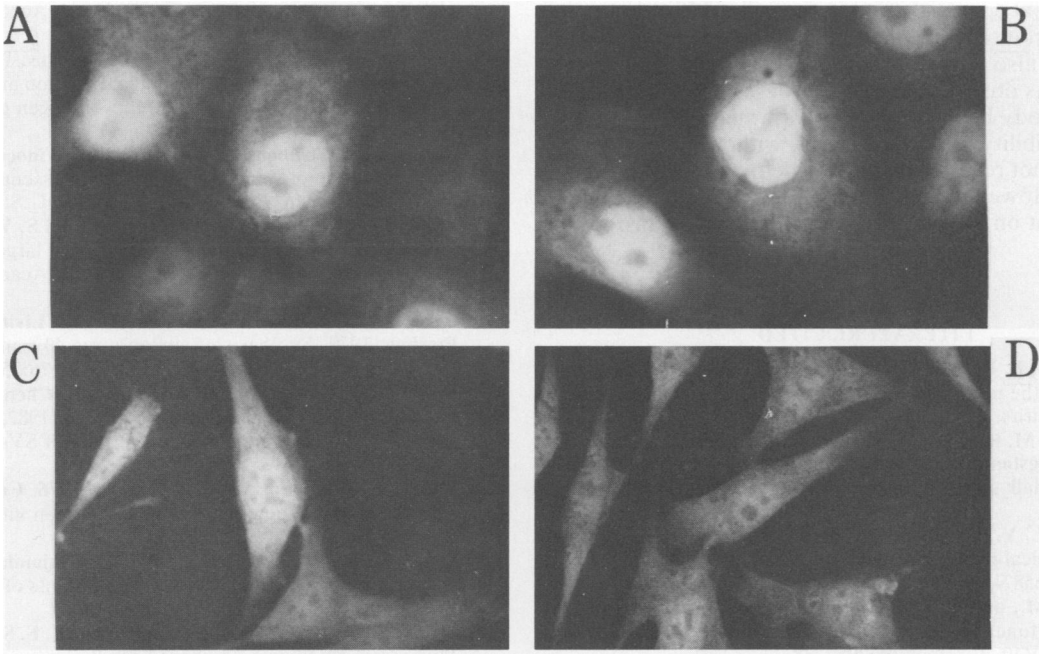

FIG. 3. Anti-t immunofluorescence analysis of SV402-infected CV-1P and NIH-3T3 clone 5-4 cells. The same adsorbed rabbit anti-t serum as that described in the legend to Fig. 2 was employed here. Cells were fixed and reacted with antibody, as noted previously (see legend to Fig. 2). SV402-infected CV-1P cells were viewed at a ×63 magnification (top panels), and SV402-infected clone 5-4 cells were viewed at a ×40 magnification (bottom panels).

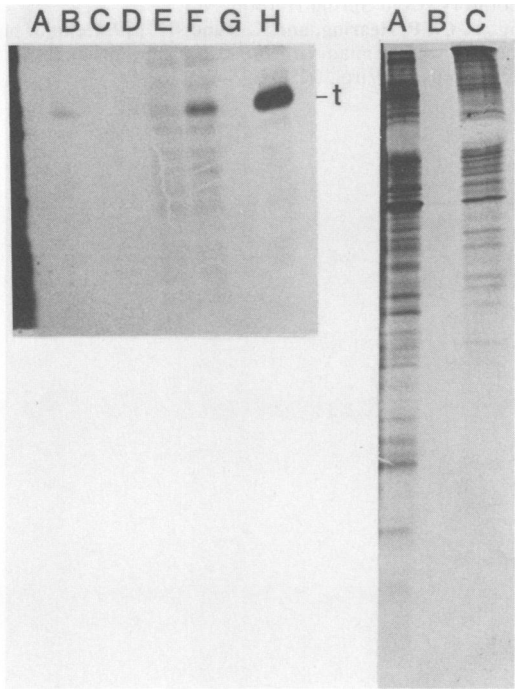

FIG. 4. Anti-t immunoprecipitation of various ^{35}S-labeled subcellular fractions of clone 5-4 cells. Two confluent, 100-mm dishes of cells were labeled for 3.5 h with [^{35}S]methionine (60 μCi/ml), and, after washing, were lysed with 1.2 ml of buffer A (50 mM Tris [pH 7.4], 1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 1 mg of bovine serum albumin per ml). After centrifugation, the supernatant was labeled "extract A" (whole-cell lysate). Cells on the other dish were scraped into 1.2 ml of buffer B (10 mM morpholineethanesulfonic acid [pH 6.2], 80 mM NaCl, 1.5 mM $MgCl_2$, 1 mg of bovine serum albumin per ml), swollen on ice for 40 min, and homogenized with 25 strokes in a Dounce homogenizer, leading to breakage of ≥95% of the cells as defined by phase-contrast microscopy. After centrifugation at 1,600 rpm for 10 min at 4°C in a Beckman J6B centrifuge, the supernatant was labeled "extract B" (postnuclear soluble fraction). The pellet was suspended in 1.2 ml of buffer B, and after extensive, gentle mixing, this fraction was again centrifuged as noted above. The supernatant was labeled "extract C" (nuclear wash). The pellet, composed of ≥95% intact nuclei, was dissolved in 1.2 ml of buffer A to yield "extract D" (nuclear extract). All fractions were centrifuged as noted above, and a 25-μl portion of each was removed for direct SDS-gel electrophoretic analysis (right panel). The remainder was immunoprecipitated with either rabbit anti-SV40 t serum or preimmune rabbit serum, as noted previously (13). Siliconized plastic centrifuge tubes immersed in a 1-mg/ml solution of bovine serum albumin for 20 min before use were employed throughout. Left panel. Lanes B, D, F, and H: anti-t serum precipitates; lanes A, C, E, and G: preimmune rabbit serum precipitates. Extracts employed were as follows: extract A (lanes G and H); extract B (lanes E and F); extract C (lanes C and D); and extract D (lanes A and B). Right panel. Direct electrophoretic analysis of the following: A, extract B; B, extract C; and C, extract D.

Ecogpt; (C and H) *dl*883-infected CV-1P cells; (D and I) SV402-infected CV-1P cells; (E and J) uninfected CV-1P cells. The rabbit serum employed was the same one used in the experiments described in the legend to Fig. 1 and was serially adsorbed at a 1:1 dilution (in PBS) against CH_3OH-fixed monolayers of NIH-3T3 cells (five successive incubations of 1 h at 23°C with confluent monolayers seeded on 60-mm plates).

function(s) or at a special point in the cell cycle or both; and (v) in addition, it remains possible that the distribution of t in a cell which also contains functional T might be different from what was observed in the cells described here. Once t-specific antibody becomes available, it might be possible to test this possibility directly. Clearly, without more information, one cannot readily conclude which, if any, of the above are correct. However, resolution of these possibilities might shed new light on how t functions in the SV40-transforming

LITERATURE CITED

1. **Abrams, H., L. Rohrschneider, and R. Eisenman.** 1982. Nuclear location of the putative transforming protein of avian myelocytomatosis virus. Cell **29**:427–439.
2. **Bikel, I., T. M. Roberts, M. T. Bladon, R. Green, E. Amann, and D. M. Livingston.** 1983. Purification of biologically active simian virus 40 small tumor antigen. Proc. Natl. Acad. Sci. U.S.A. **80**:906–910.
3. **Crawford, L. Y., and P. Z. O'Farrell.** 1979. Effect of alkylation on the physical properties of simian virus 40 T-antigen species. J. Virol. **29**:587–596.
4. **Fluck, M. M., and T. L. Benjamin.** 1979. Comparisons of two early gene functions essential for transformation in polyoma virus and SV40. Virology **96**:205–228.
5. **Gluzman, Y.** 1981. SV40-transformed simian cells support the replication of early SV40 mutants. Cell **23**:175–182.
6. **Khoury, G., P. Gruss, R. Dhar, and C.-J. Lai.** 1979. Processing and expression of early SV40 mRNA: a role for RNA conformation in splicing. Cell **18**:85–92.
7. **Martin, R. G., V. P. Setlow, C. A. F. Edwards, and D. Vembu.** 1979. The roles of the simian virus 40 tumor antigens in transformation of Chinese hamster lung cells. Cell **17**:635–643.
8. **Mulligan, R., and P. Berg.** 1981. Selection for animal cells that express the Escherichia coli gene coding for xanthine-guanine phosphoribosyl-transferase. Proc. Natl. Acad. Sci. U.S.A. **78**:2072–2076.
9. **Pipas, J. M., K. W. C. Peden, and D. Nathans.** 1983. Mutational analysis of simian virus 40 T antigen: isolation and characterization of mutants with deletions in the T-antigen gene. Mol. Cell. Biol. **3**:203–213.
10. **Prives, C., E. Gilboa, M. Revel, and E. Winocour.** 1977. Cell-free translation of simian virus 40 early messenger RNA coding for viral T antigen. Proc. Natl. Acad. Sci. U.S.A. **74**:457–462.
11. **Reddy, V. B., S. Tevethia, M. Tevethia, and S. Weissman.** 1982. Nonselective expression of simian virus 40 large tumor antigen fragments in mouse cells. Proc. Natl. Acad. Sci. U.S.A. **79**:2064–2067.
12. **Roberts, T., I. Bikel, R. Yocum, D. M. Livingston, and M. Ptashne.** 1979. Synthesis of simian virus 40 t antigen in *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. **76**:5596–5600.
13. **Rubin, H., J. Figge, M. T. Bladon, L. B. Chen, M. Ellman, I. Bikel, M. P. Farrell, and D. M. Livingston.** 1982. Role of small t antigen in the acute transforming activity of SV40. Cell **30**:469–480.
14. **Shenk, T. E., J. Carbon, and P. Berg.** 1976. Construction and analysis of viable deletion mutants of simian virus 40. J. Virol. **18**:664–671.
15. **Silver, J., B. Schaffhausen, and T. L. Benjamin.** 1978. Tumor antigens induced by nontransforming mutants of polyoma virus. Cell **15**:485–496.
16. **Sleigh, M. J., W. C. Topp, R. Hanich, and J. F. Sambrook.** 1978. Mutants of SV40 with an altered small t protein are reduced in their ability to transform cells. Cell **14**:79–88.
17. **Spangler, G. J., J. D. Griffin, H. Rubin, and D. M. Livingston.** 1980. Identification and initial characterization of a new low-molecular-weight virus-encoded T antigen in a line of simian virus 40-transformed cells. J. Virol. **36**:488–498.
18. **Tooze, J. (ed.).** 1980. DNA tumor viruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
19. **Yang, Y.-C., P. Hearing, and K. Rundell.** 1979. Cellular proteins associated with simian virus 40 early gene products in newly infected cells. J. Virol. **32**:147–154.