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# Simian Virus 40 Small-t Protein Is Required for Loss of Actin Cable Networks in Rat Cells

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The ability of the two early simian virus 40 (SV40) coded proteins, the large and small T-antigens, to abortively induce the disappearance of cytoplasmic actin-containing networks in cultured cells has been studied in rat embryo fibroblasts after microinjection of intact SV40 DNA, DNA fragments from the early region of SV40, and a purified SV40 large T-antigen related protein (the D2 hybrid protein) isolated from cells infected with the adenovirus-SV40 hybrid virus $Ad2^{+}D2$. Injection of either the 107,000-dalton D2 hybrid protein or SV40 DNA from the deletion mutant *dl* 884 SV40, which lacks part of the region (0.54 to 0.59) encoding small t-antigen, failed to cause any detectable change in the structure of actin cables in recipient cells over a period of 72 h. By contrast, injection of wild-type SV40 DNA or a DNA fragment containing the entire region coding for a small-t antigen leads to the disruption of actin cable networks within 24 h of injection. It appears likely that the SV40 small-t protein is necessary for the abortive loss of actin cables in injected cells. Epidermal growth factor also causes loss of actin cables in rat embryo fibroblasts or Rat 1 cells (an established rat embryo line), but only after exposure of the cells to epidermal growth factor in the culture medium and not after injection of epidermal growth factor into the cells.

Simian virus 40 (SV40) is a small DNA virus which is capable of transforming a variety of mammalian cells in culture (for a review see reference 33).

Recent work indicates that the 96,000-dalton "large-T" and the 17,000-dalton "small-t" proteins of SV40, both of which are encoded within the early region of the virus (5, 28, 31), are present in most SV40-transformed cell lines (34). The region coding for large-T extends from coordinates 0.655 to 0.17 on the conventional SV40 map; small-t is encoded between 0.655 and 0.54 (5, 23, 30, 31). Sequences corresponding to the region between coordinates 0.54 and 0.59 are spliced out of large-T mRNA (2) so that SV40 deletion mutants lacking sequences between 0.54 and 0.59 (*dl* 54/59 mutants) produce a normal large-T protein but either lack small-t entirely or produce a small-t protein of reduced size (5, 31).

In addition to the expression of these viral antigens, cells which have been transformed by SV40 characteristically gain the ability to produce clonal lines, to grow without anchorage, and to activate the serum zymogen plasminogen to plasmin (26). Often transformants also lose the well-developed cytoplasmic actin-containing cables characteristic of many untransformed fibroblasts. This change is a common feature of cells transformed by various DNA and RNA tumor viruses as well as by chemical carcinogens (8, 24, 29). Potent mitogens, such as plasmin or trypsin, and growth factors, for instance epidermal growth factor (EGF), also produce a rapid loss of the actin cable structure in untransformed cells (25, 34).

The role played by these cytoplasmic actin-containing networks in cellular regulation and proliferation remains obscure. However, these networks appear to play an important role in cellular adhesion, mobility, morphology, and the organization of the cell surface (1, 9, 16, 20, 22, 35, 37). It is likely that the dispersal of the actin cable network is profoundly related to loss of cellular growth regulation and transformation.

We have employed a technique involving injection of macromolecules into single rat embryo fibroblast cells (REF) in culture to study the role of the SV40 large-T and small-t proteins in the dispersal of the actin cable networks (11, 12). We find that injection of Hirt supernatant SV40 DNA or restriction endonuclease-generated or -cloned SV40 DNA fragments containing the sequences encoding an intact small-t protein, but only a substantially deleted large-T, induces the abortive disappearance of actin cables in recipient cells. Injection of a purified large-T

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related protein (the D2 hybrid protein [32]), of SV40 large-T purified from SV80 (an SV40-transformed human cell line), or of DNA from a deletion mutant of SV40 (*dl* 884), incapable of maturing small-t mRNA (18) but encoding an apparently normal large-T protein (5, 31), had no effect within 72 h of injection on the cytoskeletal structure of the recipient cells as determined by immunofluorescent staining. Thus the abortive disappearance of actin cables seems to depend on the small-t but may not require the large-T protein of SV40.

A loss of actin cables also occurs in an established rat embryo fibroblast line (Rat 1) after external exposure of the cells to EGF, but not after injection of EGF into the cytoplasm of these cells.

## MATERIALS AND METHODS

**Cells used, virus propagation, and DNA extraction.** Actin cable structure was studied in Rat 1 cells, an established Fisher rat embryo line described previously (27), and in rat embryo fibroblasts (REF) between passage numbers 16 and 20. REF cells were derived from 14-day-gestation Fisher rat embryos. For microinjection experiments, cells were plated at a density of 25 to 50 cells per $mm^2$ on small glass slides (10 by 50 mm) which are subdivided into numbered square-millimeter squares (14).

SV40 strains 776 and *dl* 884 were propagated in an established line of African green monkey kidney cells (CV1) by using Dulbecco-modified Eagle medium supplemented with 5% fetal calf serum. Wild-type SV40 and *dl* 884 DNA were isolated from CV1 cells 45 h after infection by the method of Hirt (17). Supercoiled (form I) DNA was prepared by centrifugation to equilibrium in CsCl gradients containing ethidium bromide (200 μg/ml). Ethidium bromide was extracted from the DNA I band with isopropanol (CsCl saturated) and then dialyzed against 0.01 M Tris-hydrochloride (pH 7.9)-0.001 M EDTA.

**Preparation of DNA fragments.** A 50-μg portion each of wild-type and *dl* 884 DNA was digested with 50 U of restriction endonucleases from *Haemophilus parainfluenzae* I and II (*Hpa*I and *Hpa*II, purchased from New England Biolabs; 1 U digests 1 μg of DNA to completion within 1 h under standard conditions) in 500 μl of digestion buffer (0.001 M Tris-hydrochloride [pH 7.4], 0.01 M $MgCl_2$, 0.006 M KCl, 0.001 M dithiothreitol, and 0.1% bovine serum albumin) for 2 h at 37°C. The mixture was deproteinized by extraction with 1 volume of chloroform-isoamylalcohol 24:1 (vol/vol), and DNA fragments were precipitated from the aqueous phase with 2 volumes of ethanol and 0.1 volume of 1 M NaCl at −20°C.

Fragments were separated on horizontal 1.4% agarose slab gels without ethidium bromide (electrophoresis buffer: 50 mM boric acid, 50 mM Tris base, and 1 mM EDTA [pH 8.2]). Control areas of the gels were cut out and stained with 0.5 mg of ethidium bromide per 100 ml in E-buffer for 30 min, and the gel segments corresponding to the appropriate DNA fragment bands were cut from the preparative gels. DNA fragments were removed from the gels by electrophoresis inside dialysis bags in E-buffer and filtered through a 0.22-μm sterile membrane filter (Swinnex 3; Millipore Corp.). The fragments were precipitated by the addition of 1/10 volume of precipitation buffer (0.1 M Tris-hydrochloride [pH 7.6]-0.2 M $MgCl_2$-3.5 M NaCl) and 3 volumes of ethanol at −70°C, further purified by a second round of agarose gel electrophoresis, and isolated as described above. For microinjection, the fragments were brought to a final concentration of 0.5 mg/ml of injection buffer (0.01 M Tris-hydrochloride [pH 7.6]-0.001 M EDTA) (21).

**Microinjection procedure.** Cells grown on glass slides were microinjected with about $1 \times 10^{-11}$ to $2 \times 10^{-11}$ ml of injection solution each with our microglass capillary injection technique (14).

**Detection of T-antigen and actin cables.** SV40 T-antigen and actin cable structure were visualized by the indirect immunofluorescence technique (19). Cells were fixed in 3.5% formaldehyde for 10 min, washed twice in phosphate-buffered saline, air dried, and further incubated in acetone-methanol (2:1 [vol/vol]) for 10 min at −20°C. Anti-SV40 tumor serum used was of hamster origin, and the second antibody was goat anti-hamster fluoresceine conjugated. Anti-actin was of rabbit origin (gift of Keith Burridge), and the goat anti-rabbit was rhodamin B conjugated (both second antisera are Cappel Laboratories products). The staining procedure was previously described in detail (13).

## RESULTS

**Correlation of T-antigen and actin patterns in cells injected with wild-type SV40 DNA.** When nonpermissive cells such as REF are infected with SV40 (100 plaque-forming units/cell) approximately half of the cells display a positive immunofluorescence for T-antigen 24 h after infection. At the same time, 50% of those showing viral T-antigen also lose their characteristic actin cable structures, as determined by indirect immunofluorescence using anti-actin sera. Thus, there appears to be a correlation between the synthesis of T-antigen(s) and the loss of cytoplasmic actin cable network (R. Pollack, personal communication).

A more efficient infection can be obtained by injecting viral DNA directly into REF cells. Table 1 summarizes the results obtained by microinjection of REF with various numbers of SV40 DNA molecules. When 200 to 400 DNA molecules are injected per cell, about 90% of the cells develop a positive nuclear stain for viral T-antigen during the first 24 h. This amount of DNA was used for all subsequent injections.

To test whether the actin networks of cells abortively infected by injection with SV40 DNA become disrupted in a manner analogous to that of cells stably transformed by SV40, REF cells were plated on glass slides at a density of about 25 to 50 cells/$mm^2$, and at various times after

TABLE 1. *Percentage of T-antigen-positive REF cells after microinjection of SV40 DNA I at various concentrations*[a]

| DNA concn (mg/ml) | Avg. no. of injected DNA molecules/cell | % of T-antigen-positive cells |
|---|---|---|
| 1.0 | 2,000–4,000 | 99 |
| 0.1 | 200–400 | 90 |
| 0.01 | 20–40 | 45 |

[a] Cells were fixed and stained for T-antigen 24 h after microinjection. Each value is based on a count of 100 injected cells.

microinjection, the cells were fixed and stained for T-antigen and actin (Fig. 1a).

As early as 10 h after injection, about 40% of the injected cells stained positive for T-antigen and 15% of these exhibited a significant disruption of the cytoplasmic actin cable networks. With prolonged incubation, the majority of the T-antigen-positive cells displayed a diffuse and disrupted actin cable pattern (Fig. 1a). By contrast, the actin cable structures of cells injected with buffer alone remained similar to the uninjected controls during the entire period tested.

**Injection of purified large-T antigen has no effect on actin cables.** The large T-antigen protein used for these experiments was isolated and purified to homogeneity either from HeLa cells infected with the adenovirus-SV40 hybrid virus Ad2$^+$D2 or from the SV40-transformed human cell line SV80. The defective hybrid virus contains the entire SV40 genome with the exception of the sequences mapping between positions 0.54 and 0.63. The T-antigen produced is a fused protein containing the major portion of the SV40 T-antigen covalently linked to an unidentified Ad2 polypeptide (15). The T-antigen purified from SV80 has the same electrophoretic mobility as that purified from a lytic infection. After injection of the purified D2 hybrid protein or of SV80 large-T into the cytoplasm of cells, the nuclei of injected cells showed a rapid accumulation of the antigen (Fig. 2).

In these experiments, about $5 \times 10^5$ to $1 \times 10^6$ molecules were microinjected per REF cell. Injection of $1 \times 10^5$ to $2 \times 10^5$ D2 protein molecules of this preparation per cell was sufficient to give stimulation of host cell DNA synthesis in prior experiments (32). However, neither the D2 hybrid protein (Fig. 1b and Fig. 3) nor SV80 large-T (data not shown) caused any detectable change in the actin cable structure of the recipient cells.

**Microinjection of subgenomic fragments of SV40 encoding small-t protein.** The *Hpa*I+II DNA fragment B of SV40 (map position 0.375 to 0.735; Fig. 4) contains the entire coding region for the small-t protein (Fig. 5). To obtain this fragment, SV40 DNA was first cleaved with the restriction endonuclease *Hpa*I and then by *Hpa*II as described in Materials and Methods. A characteristic nuclear fluorescence was detectable in the nuclei of injected REF cells within 24 h of injection of this fragment, due presumably to the approximately 30,000 (30K) large-T fragment which would be encoded by this DNA, and this served to identify the injected cells. These cells so identified exhibited an abortive loss of actin cable networks (Fig. 1c and Fig. 3) similar both in extent and in time scale to that which followed injection of wild-type SV40 DNA. This fragment of DNA was electrophoretically purified through two gels and was judged free of contaminating species which might encode an intact large-T because the fragment-injected cells did not stain with antisera directed against the 23K protein produced by AD2$^+$ND1.2, a protein which contains solely the 50 carboxy terminal amino acids of SV40 large-T linked to a fragment of the adenovirus type 2 fiber protein (7). Had the sequences of SV40 between 0.20 and 0.17 (containing the carboxy terminus of large-T) been present as a contaminant, a fluorescent staining with this antibody would have been observed. To further eliminate the possibility of undetected contaminating species of SV40 DNA, we carried out similar experiments using the "small Pot" plasmid, essentially PBR322 carrying an insertion of the *Hin*dIII partial digestion fragment B and C of SV40 (M. Botchan, personal communication). This cloned fragment includes the origin of transcription and the entire coding region of small-t but could encode only 180 amino acids unique to large-T. In these experiments the resulting nuclear fluorescence was very weak, but a loss of cable networks similar to that obtained with *Hpa*I$^+$II fragment B was obtained.

**Microinjection of *dl* 884 SV40 viral DNA and DNA fragments.** The early SV40 deletion mutant *dl* 884 is 3% smaller than wild-type SV40 virus, lacking a portion of the genome between 0.54 and 0.57 (30) encoding the carboxy terminus of small-t (Fig. 4 and 5) and an RNA splice point necessary to the maturation of small-t mRNA (18). REF cells injected with *dl* 884 SV40 DNA synthesize large-T antigen with approximately the same efficiency as cells injected with wild-type DNA as assayed by immunofluorescence, and cells lytically infected with the two viruses exhibit similar levels of large-T by immunoprecipitation (5, 31). However, cells injected with *dl* 884 DNA showed no loss of actin cables (Fig. 1d) within 72 h of injection. If the abortively infected cells are observed for periods of up to 100 h, some disruption of the cable patterns is observed (data not shown). Similar results were obtained

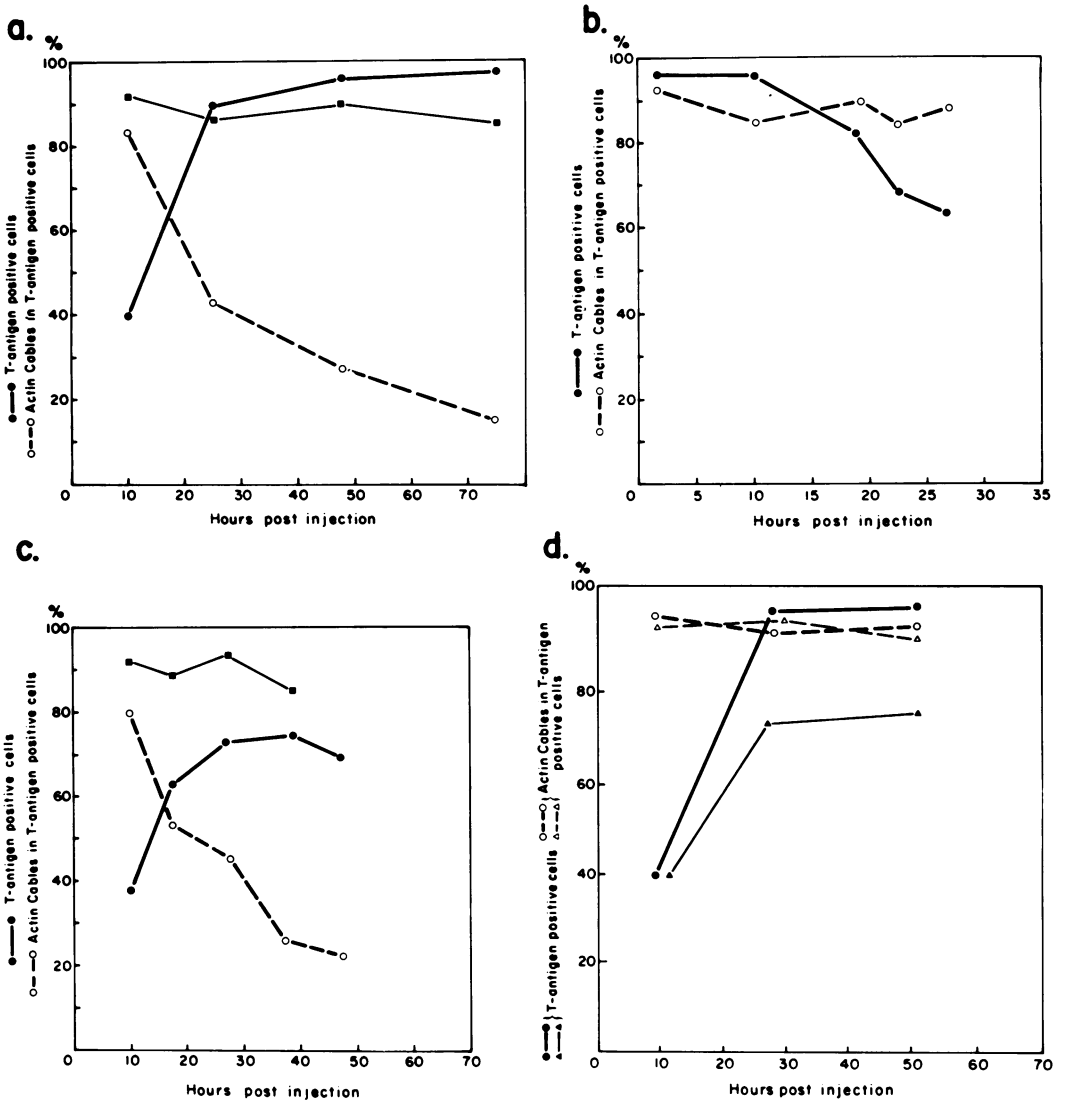


FIG. 1. *Correlation between intranuclear T-antigen accumulation and loss of actin cable structure in microinjected REF cells. (a) Microinjected with wild-type SV40 DNA. (b) Microinjected with purified D2 T-antigen. (c) Microinjected with wild-type SV40 HpaI+II fragment B DNA.* ■, *Percent of actin cable-positive cells mock injected;* ●, *percent of T-antigen-positive cells;* ○, *percent of T-antigen positive cells which retain actin cable structures. (d) Microinjected with dl 884 DNA* (●——●, ○---○) *or dl 884 HpaI+II fragment B DNA* (▲——▲, △-----△). *Only well-spread cells were used for microinjection to lower the background of actin cable-negative cells. Randomly selected REF cells are ≈10 to 20% actin cable negative. An average of 100 cells were injected for each point. All cells within a delineated well-marked portion of a slide were injected, and stainable antigens persisted for at least 72 h. Slides were frequently examined by two observers who were generally in agreement. Occasionally cells were observed in which cable patterns were plainly visible yet highly fragmented and disordered. These were scored as cable negative. Occasional cells which stained very weakly with a pattern resembling that of T-antigen were not scored since such cells occur in uninjected populations. Cells which seemed to be just entering or recovering from mitosis were also not scored.*

after injection of the *Hpa*I+II DNA fragment B (Fig. 1d) from *dl* 884.

**EGF and loss of actin cables.** For the experiments involving EGF and loss of actin cables, we used Rat 1 cells instead of REF cells because this cell line exhibited a stronger and more consistent response to EGF. We have shown that at a concentration of 30 ng of EGF

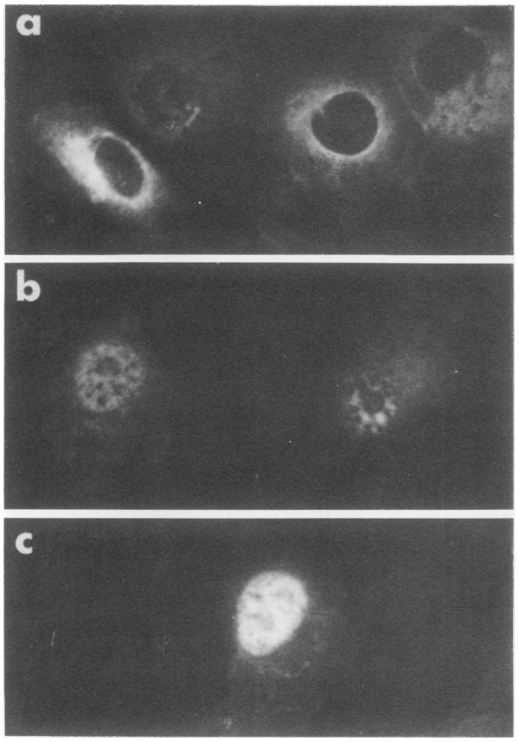


FIG. 2. *Intranuclear T-antigen accumulation. Purified D2 T-antigen molecules were microinjected into the cytoplasm of recipient cells. Cells were fixed and stained with anti-T serum at various times (a, immediately; b, 30 min; and c, 2 h) after microinjection.*

per ml in culture medium about 90% of Rat 1 cells lose actin cables during the first 24 h (34).

To test whether EGF can also disrupt the actin cable networks when injected into the cytoplasm of Rat 1 cells, the growth factor was injected either into the cytoplasm or nuclei of the recipient cells. Injection of up to $5 \times 10^6$ to $10 \times 10^6$ EGF molecules per cell produced no reduction of actin cable structures (Table 2). REF cells were similarly unresponsive upon injection of EGF (data not shown).

To test whether the inability of EGF to alter actin cable structures when injected into cells is due to some toxic effect, we repeated the injection experiments with SV40 DNA in the presence of added EGF. The growth hormone did not interfere with the effect of SV40 on actin cables (Table 2).

## DISCUSSION

REF cells transformed by SV40 fall into two distinct categories, the maximal and the minimal transformants (26). The maximal transformants show anchorage-independent growth, a low serum requirement, a loss of actin cable structures, serum plasminogen activation, and tumor formation in nude mice (8, 24, 26). Minimal transformants have only a reduced serum requirement and the ability to clone on plastic dishes. Thus far, only two virus-coded proteins, the large (96K) and small (17K) T proteins have been identified in cells transformed by SV40. The relative roles played by these two proteins in transformation are of interest to us.

Viral mutants exist which are useful in analyzing these problems. Early *dl* 54/59 SV40 mutants plaque on CV1 cells, although many of these mutants (for instance *dl* 884) give a reduced viral burst relative to wild-type SV40 (33a). The *dl* 54/59-infected cells synthesize a normal large-T antigen, but the small-t antigen is either reduced in size or entirely absent (5, 31). This suggests that small-t antigen is not absolutely required for viral growth. However, REF cells transformed by *dl* 884 SV40 virus fall predominantly into the class of minimal transformants (3, 31) and retain their well-articulated actin cable networks (Topp and Rifkin, manuscript in preparation). This suggests that small-t activity is involved in the loss of actin cables.

Indeed it is clear from the experiments presented here that neither the D2 hybrid protein, the SV80 T-antigen, nor the wild-type large T-antigen of *dl* 884 alone is sufficient to abortively alter the actin cable structure. Only at greatly prolonged times did *dl* 884 DNA induce even a partial abortive cable loss, and no reduction was ever seen after injection of the purified viral proteins. In all cases the bright nuclear fluorescence characteristic of viral T-antigen was observed, and, as shown in previous experiments, these proteins are biologically active when introduced in this manner (32). By contrast, injection of wild-type SV40 DNA or a DNA fragment encoding only the small-t protein (*Hpa*I+II fragment B map position 0.735 to 0.375 or small Pot plasmid) induced the abortive disappearance of cytoplasmic actin cables. This activity strongly suggests that the small-t protein is required for the loss of actin cables. (A similar result for polyoma virus has recently been reported [29].) We are unable however to conclude from these studies that it is the small-t protein alone which is responsible for the abortive cable loss we observe. Both the *Hpa*I+II fragment B and the small Pot plasmid contain some sequences encoding the amino-terminal portion of large-T. Our data are equally consistent with a coordinate mechanism of action which would involve both the small-t and this large-T fragment, although we have never observed any large-T-associated functions by microinjection of this

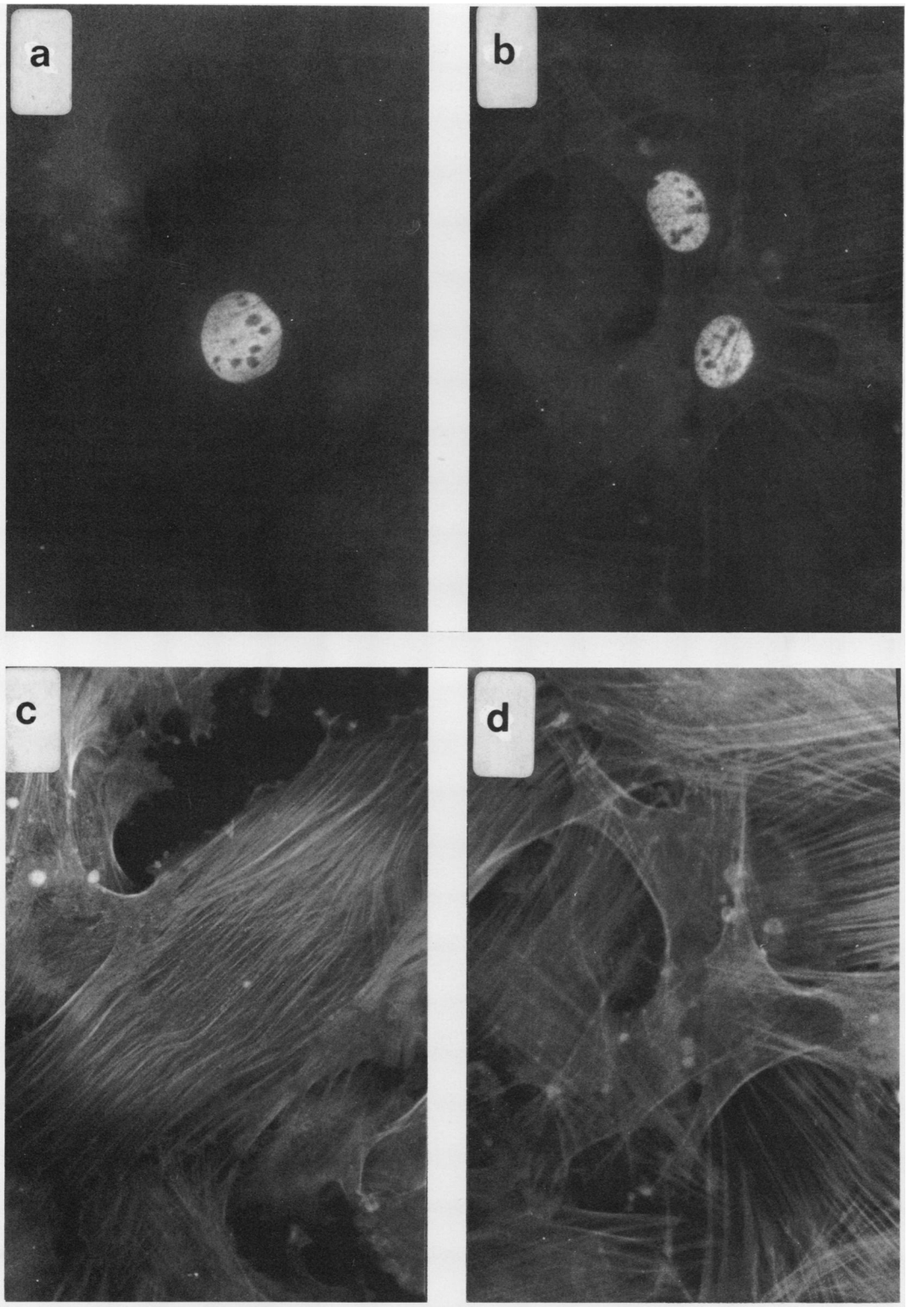


FIG. 3. *Parallels between intranuclear T-antigen staining and cytoplasmic actin cable structures in microinjected REF cells. (a) D2 T-antigen microinjected cells. (b) T-antigen staining in wild-type HpaI+II fragment B DNA-injected cell. (c) Actin cable structure in the D2 T-antigen-injected cell. Magnification, ×800. Cells were fixed and stained 24 h after microinjection. (d) The same cell as in (b) except stained with anti-actin.*

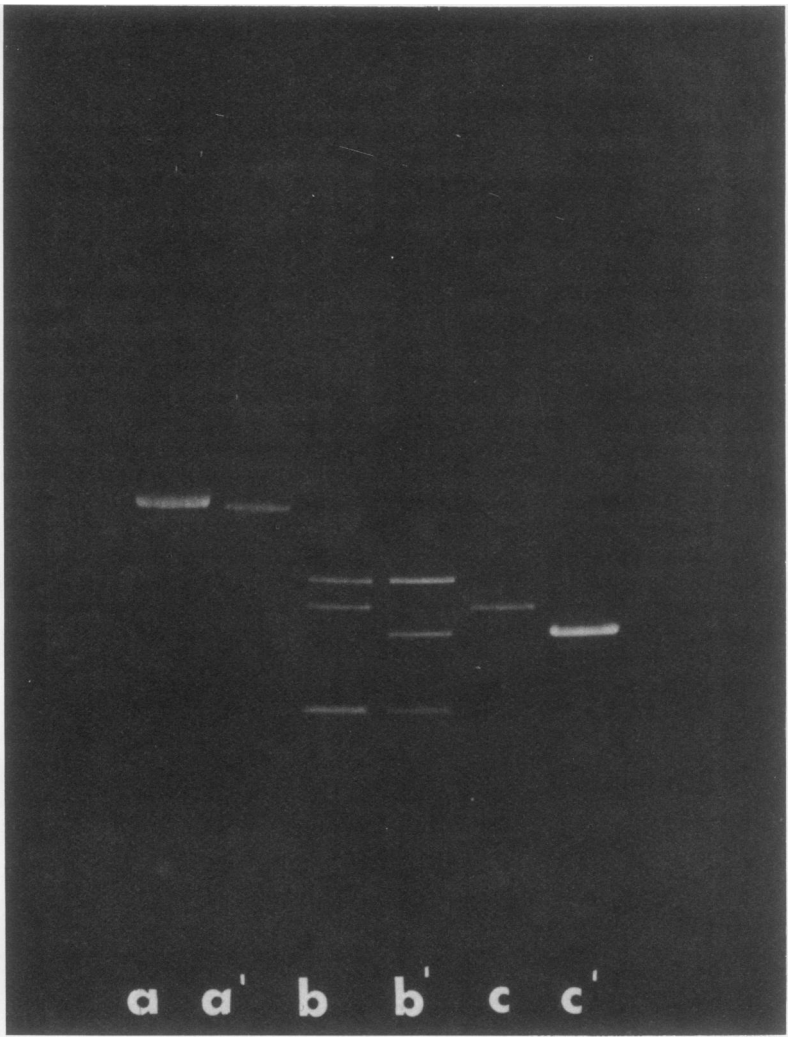


FIG. 4. *Agarose slab gel electrophoresis of SV40 DNA and DNA fragments. A 0.2-μg amount each of DNA and DNA fragment was subjected to electrophoresis on a 1.4% agarose slab gel. The gel was stained with ethidium bromide and photographed under short-wave UV illumination with a Kodak no. 23A red filter. (a) Wild-type DNA I and II; (b) wild-type HpaI+II fragment A, B, C DNA; (c) wild-type HpaI+II fragment B DNA. dl 884 DNA and DNA fragments are in the same order except they are indicated by a', b', and c'.*

fragment (2). It will be necessary to purify the small-t protein to settle this issue.

Several papers have been published reporting the temperature sensitivity of actin cable networks in *tsA* SV40 transformants, suggesting a dependence of actin cable networks on large-T (24, 36). If the loss of actin networks is dependent on large-T, then our results suggest that those sequences of large-T contained in *Hin*dIII fragment B and C (small Pot) are involved. On the other hand, if small-t is ultimately found to be solely responsible for our abortive loss of cables, then there is an apparent contradiction between our data and those which precede it. In one of these studies (24) a substantial fraction of the cells (*tsA* SV40 transformants) lost SV40 viral T-antigen and became actin cable positive as assayed by immunofluorescence at the restrictive temperature. Return of the T-antigen staining pattern on downshift required RNA synthesis (Topp, unpublished data) and it is likely that the viral genome was no longer being transcribed at the restrictive temperature. A disappearance of small-t antigen in these cells at the nonpermissive temperature is to be expected. In another previous work (36) 100% of the cells remained T-antigen positive by immunofluorescence at the restrictive temperature (J. Butel,

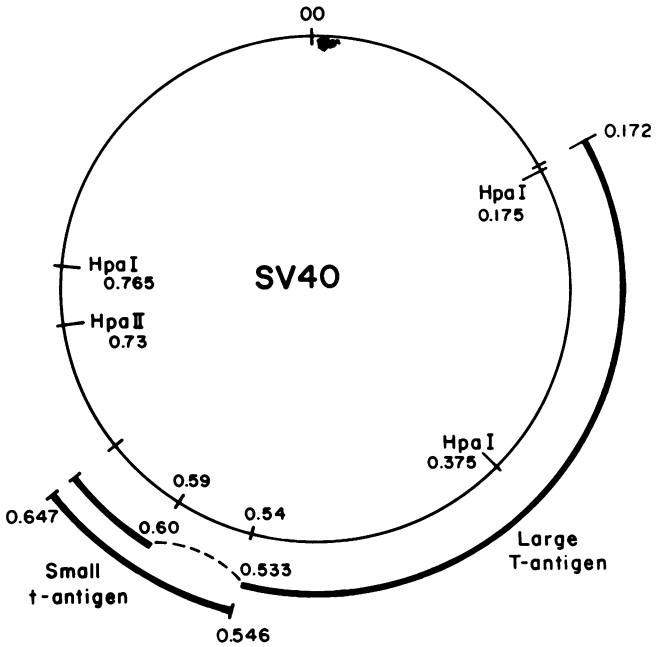


FIG. 5. *Physical map of SV40 with the cleavage sites of the restriction endonucleases HpaI and HpaII. The coding region for large- and small-T proteins, the region spliced out from the large-T mRNA (0.533 to 0.60), and the deleted region of the 884 virus (0.535 to 0.58) are also indicated.*

TABLE 2. *Change of cytoskeletal structure in microinjected REF cells*

| Material injected into cells | Nuclear T-antigen staining | Reduction of cytoplasm actin cables |
|---|---|---|
| T-antigen (D2 hybrid protein) | + | − |
| Wild-type DNA *HpaI+II* fragment B | + | + |
| EGF[a] into cytoplasm | − | − |
| EGF[a] into nuclei | − | − |
| EGF + T-antigen | + | − |
| EGF + wild-type DNA *HpaI+II* fragment B | + | + |
| EGF added to the medium (25 ng/ml) | ND | + |

[a] At the injection conditions used, $5 \times 10^6$ EGF molecules (Collaborative Research Inc.) were microinjected per recipient cell. Cells were fixed and stained 6, 12, 24, 36, and 48 h after microinjection. Similar results were obtained when Rat 1 cells were used for microinjection. ND, Not done.

personal communication). In this study cables were visualized by electron microscopy, and it is possible that the immunofluorescent assay that we have employed exhibits a different set of structures from that pictured in electron microscopy. Such an effect has been reported previously (10). We feel that none of the results from these experiments is irreconcilable with our results at this time.

The ultimate goal of these experiments is to provide some clue to the mechanism of action of these viral proteins. Recent experiments on REF cells transformed by *dl* 54/59 and *tsA* SV40 mutants have shown that the activation of plasminogen to plasmin is also primarily dependent on small-t antigen (Topp and Rifkin, in preparation). It is as if the minimally transformed REF isolated after wild-type SV40 infection behave as if they are $T^+t^-$ whereas the maximal transformants are $T^+t^+$ (34).

It is of interest that the same properties, growth in methocel, plasminogen activation, and loss of actin cables, which we associate with small-t protein, all have been separately observed to follow exposure of cells to EGF and other similar growth factors (34; Rifkin and Pollack, unpublished data). We have observed a $10^2$-fold increase in the efficiency of cloning in agar of an established Fisher rat embryo line (Rat 1) in the presence of 25 ng of EGF per ml. We also observe a profound morphological change accompanied by the disappearance of actin cables in approximately 90% of cells exposed to EGF for 24 h (34). An intriguing possibility is that the small-t antigen and EGF alter cellular patterns by a similar mechanism.

It is difficult to imagine a direct analogy between EGF and the SV40 small-t protein because the normal response to EGF follows exposure to this hormone at the outer cell surface, whereas small-t protein is cytoplasmically local-

ized and has not been found secreted into the culture medium (Todaro, personal communication; Sleigh and Topp, unpublished data). It is true, however, that the EGF-receptor complex is rapidly internalized after binding of EGF and eventually degraded in lysosomes (4). To investigate the possibility that EGF is actually acting inside the cell, we microinjected several million EGF molecules directly into the cytoplasm or nuclei of Rat 1 cells, a number 10-fold greater than the number of EGF receptors on most cells. No effect on actin cables was observed. The lack of EGF activity in the cell was not due to toxicity of the growth hormone because coinjection of EGF with SV40 DNA had no effect on the DNA results. Either EGF acts at the cell surface or it has to be modified before entering the cell, or the microinjected EGF failed to be properly compartmentalized.

It is unlikely that small-t protein is reducing cables by stimulating the secretion of cellular hormones in a manner analogous to the *sarc* gene of mouse sarcoma virus (Todaro and DeLarco, personal communication) because no *trans* effect of cable loss is seen (data not shown). Only cells which have been injected lose actin cables, whereas adjacent cells remain unchanged.

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