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Monoclonal Antibody to Simian Virus 40 Small t

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A monoclonal antibody, PAb280, was produced that recognizes simian virus 40 (SV40) small t but does not react with SV40 large T. The specificity of the antibody was analyzed by immunoprecipitation of labeled cell extracts, Western blotting, and immunocytochemistry. Small t was found to accumulate late in the SV40 lytic cycle and was localized in both the cytoplasm and the nucleus of cells infected with wild-type SV40. Importantly, antibodies against determinants common to SV40 large T and small t did not appear to be able to recognize the cytoplasmic form of SV40 small t at the immunocytochemical level. The localization of small t within the nucleus appeared to be distinct from that of large T.

The genome of simian virus 40 (SV40) encodes six known proteins: small t, large T, VP1, VP2, VP3 (30), and agnoprotein (19). Of these proteins, only two, small t and large T, are normally synthesized in abortively infected or transformed cells (30). In lytic infection, synthesis of small t and large T is detected before the onset of viral DNA synthesis and persists throughout the infection (6, 30). The mRNAs that code for small t and large T are thought to be derived from the same precursor RNA transcript which is differentially spliced to produce the two mRNAs such that small t and large T share an amino-terminal region of 82 amino acids but differ in their carboxy-terminal amino acid sequences. A very large number of biochemical and physical properties of large T have been described, and compelling genetic evidence exists to indicate the pivotal role of large T in both the viral lytic cycle and cell transformation (20, 30). The functions and properties of small t are far less clearly defined. Deletion mutants that affect splicing of the small t transcript, but not of the large T transcript, have been isolated. These viruses, although they synthesize a normal large T, do not synthesize any detectable small t (14). Other mutants, reflecting deletions within the large T intron, produce truncated small t antigens but, again, synthesize normal large T (5, 14). All of these mutants are viable in permissive tissue culture cells. Topp (31), however, has demonstrated that the small t deletion mutants are slightly defective in that they produce a smaller virus burst at low multiplicities of infection. This implies that small t does play some role in lytic infection in in vitro conditions. In in vivo infections, the role of small t may be more important, but this has not yet been assessed.

A large body of work has addressed the crucial issue of the role of small t in cell transformation. Initial reports have indicated that small t is extremely important, since small t deletion mutants have been shown to be partially defective for transformation (26). Subsequent studies have shown considerable variation in the different transforming potentials of wild-type and small t deletion mutant viruses (7, 18, 21, 23, 27). A somewhat simplified, consensus viewpoint is that small t is required in situations in which the normal growth of untransformed recipient cells is restricted soon after virus infection, or at the time of virus infection, by conditions that permit only the growth of transformed cells, for example, growth in semisolid media or overgrowth of the monolayer. Actively growing cells are usually transformed with near-equal efficiency by both wild-type and deletion mutant viruses. A particularly clear recent study by Rubin et al. (21) exploited transfection of a small t-encoding, but non-large T-encoding, plasmid in the presence or absence of infection with small t deletion mutant viruses to demonstrate complementation between small t and large T in both abortive and permanent transformation of BALB/c 3T3 cells as assessed by growth in semisolid medium.

The biochemical properties of small t are also less well defined than those of large T. Purified small t, which is derived from the expression of the small t gene in *Escherichia coli*, will induce partial dissociation of the actin cable network when it is microinjected into the cell cytoplasm of RAT 1 cells (2). This is a direct result mirroring the earlier observations that wild-type SV40 DNA microinjected into rat embryo fibroblasts induces actin cable disruption but that deletion mutant DNA does not (8). In this latter study, it was observed that DNA fragments capable of encoding intact small t, but only a grossly truncated large T, also induce actin cable dissociation.

Small t has been shown to specifically associate with two host proteins; one is 56 kilodaltons and one is 32 kilodaltons (22). It is also shown to be involved in the stimulation of the appearance of a host centriolar antigen (25), but the correlation of these properties to its functions is not yet clear.

Many characteristics of small t, e.g., cellular location and precise quantitation, have been hard to elucidate because of the absence of a specific antibody to the protein. It has been demonstrated originally by Lane and Robbins (17) that small t and large T share common antigenic determinants; more recently, monoclonal antibodies reactive to both large T and small t have been isolated by a number of groups (10, 11; J. Yewdell and D. P. Lane, manuscript in preparation). No antibody specific to small t has been derived from these fusions, however, despite the demonstration that antibodies of such specificity do occur, at least in hamster immune response to SV40-induced tumors (9). We set out, therefore, to exploit the existence of plasmids that direct the synthesis of small t in abundant amounts in *E. coli* to produce a monoclonal antibody which is specific to small t.

MATERIALS AND METHODS

Viruses and monoclonal antibodies. Monoclonal antibodies PAb419, PAb423, and PAb414 were a kind gift from Ed Harlow (Cold Spring Harbor Laboratory [11]).

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SV40 *dl* 883 (24) was a gift from L. V. Crawford (Imperial Cancer Research Fund). Large-plaque wild-type SV40 stock was grown in CV1 cells to a final titer of 5×10^8 PFU/ml.

CV1 and SP20/AG14 cells, as well as the hybridomas, were grown in Dulbecco modified Eagle minimum essential medium supplemented with 10% fetal calf serum and 500 U of penicillin and 100 μg of streptomycin per ml.

Extraction of small t. Small t was isolated from *E. coli* cells containing plasmids in which the small t gene had been placed under the control of strong bacterial promoters. The plasmid HP1 with the small t gene controlled by the *lac* UV-5 promoter was a gift from R. Tjian (29), and the plasmid pTR865 with the small t gene controlled by the *tac* promoter was a gift from D. Livingston (2).

Bacterial cells were grown to mid-log phase in L broth supplemented with 25 μg of ampicillin per ml, and then isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 5 mM, and the cells were allowed to grow to late log phase. The cells were then harvested, and the pellet was resuspended to 1/50 of the original volume in NET (150 mM NaCl–5 mM EDTA–10 mM Tris-hydrochloride [pH 8.0]) containing 1% Nonidet P-40 (NP-40) and 1 mM phenylmethylsulfonyl fluoride. This suspension was sonicated for 5 min, and then the extract was centrifuged at 10,000 rpm for 30 min by using a GS34 rotor in a Sorvall centrifuge; the supernatant was kept.

Purification of PAb419. Monoclonal antibody PAb419 was purified by protein A-Sepharose chromatography from the tissue culture supernatant of the hybridoma culture. The antibody was bound to the column (packed volume, 5 ml) by recycling 500 ml of the supernatant through the column for 48 h at 4°C. After the column was washed extensively with 150 mM NaCl–10 mM Tris-hydrochloride (pH 8.0), followed by sodium phosphate buffer (0.01 M [pH 8]), the antibody was eluted with citrate buffer (0.1 M citric acid–0.1 M trisodium citrate [pH 3]) as 1-ml fractions which were neutralized immediately with 1 ml of 1 M Tris-hydrochloride (pH 8.8) and stored at 4°C.

Isolation of PAb419-small t complex. Bacterial cell extract (10 ml) was mixed with 1 mg of purified PAb419 and 1 ml of protein A-Sepharose and allowed to incubate at 4°C overnight. The mixture was then poured into a 5-ml column (Bio-Rad Laboratories) and allowed to settle. The column was washed first with 50 ml of 150 mM NaCl–10 mM Tris-hydrochloride (pH 8.0), and then with 100 ml of sodium phosphate buffer (0.01 M, pH 8). Finally, 0.1 M citrate buffer (pH 3) was used to elute the complex (1-ml fractions) into 1 ml of 1 M Tris-hydrochloride (pH 8.8) which neutralized it.

Immunization. Female BALB/c mice (5 to 6 weeks old) were immunized with 10 μg of the purified PAb419-small t complex. The first two injections were given intraperitoneally in Freund incomplete adjuvant. The mice were boosted before fusion by intravenous injection of the purified complex in saline. Serum samples were monitored for the presence of antibodies to small t by staining cultures of CV1 cells infected with wild-type or *dl* 883 viruses.

Hybridoma fusion. The method for hybridoma fusion was basically that described by Kennett et al. (13). The spleen cells of the immunized mice were suspended in serum-free medium and fused to 10^7 SP20/AG14 myeloma cells by using polyethylene glycol. The fusions were plated out among 800 to 1,000 separate wells, and growing hybrids were obtained at frequencies of between 100 to 500 events per fusion.

Cloning and growth of hybridoma cells. The hybridoma cells from wells that gave a positive signal in the screening assays were transferred to 24-well Costar trays, and after growing for 3 to 4 more days, the cells were cloned by using the soft agarose method (13). Agarose colonies were picked after 8 to 10 days; individual colonies were rescreened, recloned, retested, and finally grown in bulk and frozen at 10^7 cells per ml in liquid nitrogen in a freezing mixture consisting of 5% dimethyl sulfoxide–95% serum. Recovery of frozen cells was usually excellent under these conditions. For growth as ascitic tumors, 10^6 hybridoma cells were inoculated intraperitoneally into pristane-primed BALB/c mice (0.2 ml of pristane oil was given intraperitoneally 10 days earlier).

Solid-phase radioimmunoassay for hybridoma screening. Of bacterial extracts prepared from *E. coli* synthesizing small t or control cells, 25 μl was added to each well of a 96-well, flexible, plastic microtiter plate (Flow Laboratories, Inc.) and allowed to dry overnight at 4°C. Of each hybridoma supernatant tested, 25 μl was added to each well, and the plate was incubated for 2 h at room temperature. The plates were washed five times in phosphate-buffered saline (PBS), and then 25 μl (50,000 cpm) of iodinated sheep anti-mouse immunoglobulin (Amersham Corp.), diluted in PBS containing 5% fetal calf serum, was added to the wells. After 1.5 h of incubation at room temperature, the plate was washed as described above, dried, and cut up with a hot wire machine. The individual wells were counted in a gamma counter.

Gel electrophoresis and Western blotting. Polyacrylamide gels (15%) were prepared by the method of Laemmli (16). The amounts of protein loaded in each gel are as described in the figure legends.

For Western blotting (32), protein bands from a sodium dodecyl sulfate (SDS)–15% polyacrylamide gel were transferred onto nitrocellulose paper (Schleicher & Schuell, Inc.) for 2 h at 1 A by using an electroblot apparatus (E-C Apparatus Corp.). The papers were exposed on RX Fuji X-ray film with a Fuji Mach II intensifying screen for 6 h at −70°C to detect the radioactive markers run with every gel.

The blot was first incubated overnight at 4°C with 5 ml of antibody-containing tissue culture supernatant from the hybridoma cells. After being washed five times over a period of 50 min in PBS (150 mM NaCl, 30 mM NaH_2PO_4 [pH 7.2]) containing 1% NP-40, the blot was incubated overnight at 4°C in 5 ml of a 1:100 dilution of horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin. Finally, the blot was washed twice in PBS–1% NP-40, once in PBS–0.1% NP-40, and three times in PBS alone. The blot was developed with a 4-chloro-1-naphthol substrate solution (saturated solution in ethanol diluted 1/100 in PBS and filtered through a Whatman no. 1 filter paper, and then H_2O_2 [30 volumes of stock] was added at a 1/5,000 dilution).

Immunoprecipitation. CV1 cells (10^7) infected with wild-type SV40 or with *dl* 883 for 72 h were labeled for 3 h with 50 μCi of [^{35}S]methionine in methionine-free medium to give a specific activity of 10^7 cpm per ml of cell extract. The cells were extracted in 0.5 ml of buffer (0.1 M NaCl, 0.05 M Tris [pH 7.9], 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1% NP-40) and centrifuged at 10,000 rpm for 30 min. To the supernatant, 15 μl of normal rabbit serum was added and left to incubate for 1 h at room temperature; then 50 μl of 10% Formalin-fixed *Staphylococcus aureus* was added, and the mixture was incubated at 4°C for 15 min more. The last step was repeated twice. One-third of the final cell extract volume was mixed with 2.5 μg of PAb280 or PAb419 and incubated overnight at 4°C, and then 50 μg of rabbit anit-mouse immunoglobulins (DAKO Corp.) was added, and the mixture was incubated further for 2 h at room temperature. Finally, 100 μl of 10% Formalin-fixed *S. aureus* was added,

and the mixture was incubated for 1 h at 4°C. After this period, each immunoprecipitation reaction was centrifuged for 30 s (Eppendorf microfuge). The supernatant was discarded, and the pellet was then washed once with each of the following buffers: (i) 50 mM Tris (pH 7.4)–1% NP-40–0.1% SDS–100 mM NaCl, (ii) 50 mM Tris (pH 8)–1% NP-40–600 mM NaCl, (iii) 50 mM Tris (pH 8)–1% NP-40–0.1% SDS–0.5 M LiCl, (iv) 150 mM NaCl–5 mM EDTA–50 mM Tris (pH 7.9)–0.05% NP-40–1% bovine serum albumin, (v) 150 mM NaCl–5 mM EDTA–50 mM Tris (pH 7.9). After the last wash, the pellet was resuspended in 2× sample buffer (16), boiled for 2 min, and centrifuged for 1 min, and the supernatant was run on an SDS–20% polyacrylamide gel.

Horseradish peroxidase staining. CV1 cells, 72 h after infection with wild-type SV40 or *dl* 883 or after mock-infection, were fixed on the plates for 15 min with 3% paraformaldehyde in 85% PBS (in water) (pH 7.4) with 1 mM Ca^{2+}-Mg^{2+} at 4°C, rinsed twice with 85% PBS, and further incubated with 50 mM NH_4Cl in 85% PBS for 20 min at 4°C. Finally, they were permeabilized by a freeze-thaw cycle by using a dry ice–ethanol bath, rinsed again twice with 85% PBS, and incubated with purified monoclonal antibody (100 μg/ml; diluted with 85% PBS–10% fetal calf serum) overnight at 4°C. Cells were rinsed twice with 85% PBS and further incubated with rabbit anti-mouse immunoglobulin–horseradish peroxidase conjugate (DAKO) at a 1:50 dilution in PBS–10% fetal calf serum for 3 h at 4°C; the plates were rinsed as described above. An oversaturated solution of *o*-dianisidine was prepared in ethanol, and from this, a 1:100 dilution was made in PBS and filtered; H_2O_2 (30 volumes; BDH) was added to attain a 1:5,000 dilution. The plates were incubated in this substrate solution for 30 min, rinsed in distilled water, and mounted in Gelvatol (Monsanto Chemicals).

RESULTS

Isolation of small t for immunization. Although immunization of mice or hamsters with syngeneic SV40-transformed cells induces a good immune response to SV40 large T and to the common antigenic determinants of small t and large T antibodies specific to small t arise at very low frequencies (9), and no monoclonal antibodies specific for small t have been isolated from the spleens of mice immunized in this manner (10, 11; J. Yewdell and D. P. Lane, unpublished data).

To prepare a suitable immunogen, we decided therefore to take advantage of plasmids which direct the expression of small t in *E. coli*, since the quantity of small t that could be obtained from this source was expected to be much greater than that isolated from lytically infected cells. Initially, we used the plasmid HP1 described by Thummel et al. (29) in which small t is under the control of the UV-5 *lac* promoter. In our hands, the yields of small t that could be obtained from this source were disappointing since, although the bacteria transformed by HP1 synthesized small t very efficiently as judged by ^{35}S-pulse-labeling of cultures, the steady-state level of small t was low. To overcome this problem and hopefully to enhance the immunogenicity of the small t unique epitopes, we isolated the small t from soluble extracts by using a monoclonal antibody (PAb419) directed against the common determinants of small t and large T (11); we then immunized mice with the purified antibody-antigen complex. Such complexes appeared highly immunogenic, since amounts (less than 1 μg) of small t detectable only by silver staining of gels induced good antibody responses.

When plasmid pTR865 was made available to us, we were able to isolate much larger quantities of small t, since *E. coli* X90 cells transformed by pTR865 synthesize and accumulate large quantities of small t on induction with IPTG. Although Bikel et al. (2) have purified small t from pTR865-transformed *E. coli* cells by using conventional protein chemical methods, we retained our immunoaffinity protocol because of the high immunogenicity of the PAb419-small t complexes and because we hoped that it would direct the immune response to the unique epitopes of small t. The induction of small t in *E. coli* X90 cells containing pTR865 and the isolation of small t-PAb419 complexes are illustrated in Fig. 1.

Immunizations and fusions. A total of 10 fusions were carried out in mice immunized with the complex. In each fusion, between 100 and 1,000 hybrid clones arose and were screened. Although many hundreds of clones producing antibody to small t were detected by their ability to bind small t in the solid-phase radioimmunoassay, subsequent analysis of these antibodies by immunoperoxidase staining of wild-type or *dl* 883-infected CV1 cells proved that all of these clones, with only three exceptions, were directed to epitopes shared by SV40 small t and large T in that the antibodies stained the nuclei of *dl* 883-infected cells. Of the three exceptions, one antibody was successfully cloned and characterized. In accordance with the proposals of Crawford and Harlow (3), this antibody is designated PAb280.

Characterization of PAb280. PAb280 was originally identified as a putative anti-small t-specific antibody since it scored strongly in the solid-phase radioimmunoassay with extracts of *E. coli* containing small t, but not with control extracts. It shares this property with the many antibodies isolated that recognize epitopes common to large T and small t, but unlike these reagents, PAb280 failed to stain *dl* 883-

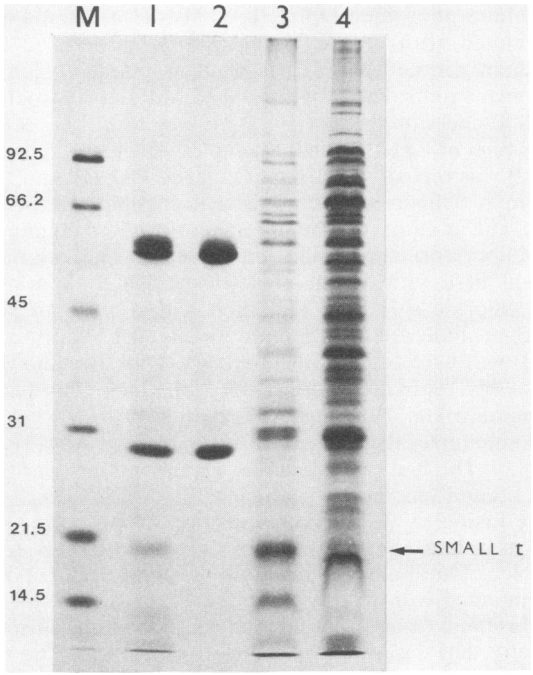

FIG. 1. Isolation of the small t PAb419 complex. Samples were analyzed by electrophoresis in an SDS–15% polyacrylamide gel. The separated proteins were visualized by staining the gel with Coomassie blue. Lane M, molecular weight markers, lane 1, 10 μg of the PAb419-small t complex; lane 2, 10 μg of PAb419 alone; lane 3, 20 μg of a total cell extract of *E. coli* X90 cells containing pTR865 after induction with IPTG; lane 4, 20 μg of a whole-cell extract of *E. coli* X90 cells containing pTR865 in the absence of induction.

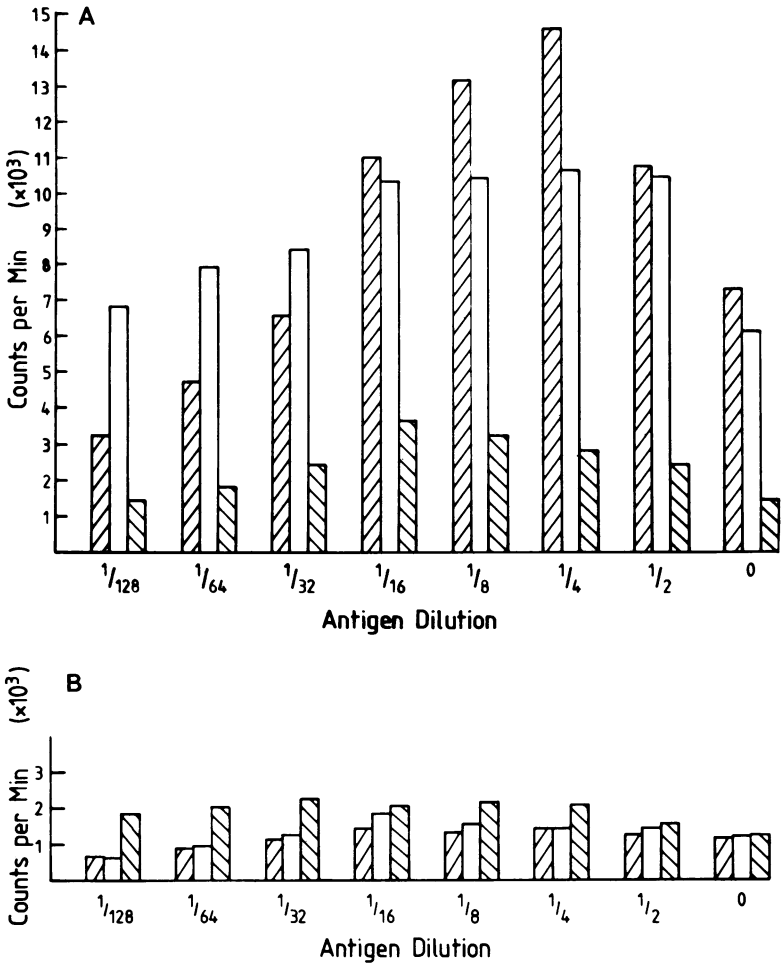

FIG. 2. Solid-phase radioimmunoassay demonstrating the specificity of PAb280 for small t. Bacterial cell extracts were prepared from *E. coli* X90 cells containing pTR865 induced with IPTG (A) or from X90 control cells containing no plasmid (B). Serial dilutions (25 μl) of the two extracts were plated into 96-well microtiter plates and dried onto the plates by overnight incubation at 37°C. The binding of PAb280 (□), PAb419 (▨), and PAb414 (▧) (11) (an antibody that binds large T but not small t) to the cell extracts was determined with iodinated sheep anti-mouse immunoglobulin as described in the text. The hybridomas were used as undiluted tissue culture supernatants.

infected CV1 cells. The cells producing PAb280 were therefore cloned twice through soft agarose, and the individual clones were analyzed for reactivity to small t. All the clones picked after both the first and second rounds of cloning retained the two properties of the parent cells. PAb280 binding to small t-containing extracts of *E. coli* is illustrated in Fig. 2. It is clear that PAb280, like the control anti-large T-small t-reactive antibody PAb419, recognized something present in an extract of induced X90 cells containing pTR865 but showed no specific binding to the same cells lacking the plasmid. The solid-phase assay proved to be very reliable and ideally suited to screening large numbers of samples. It was found to be important that mice were immunized with the complex in Freund incomplete adjuvant, as the mycobacteria present in the complete adjuvant induced a strong antibacterial response which gave rise to a large number of hybridomas which secrete antibodies that react with all *E. coli* extracts. When the solid-phase assay was used at a fixed-antigen concentration (1/4; Fig. 2) to determine titers in tissue culture supernatants from both PAb419 and PAb280 cells, both antibodies had a titer (50% binding of plateau level) of approximately 1/128. Ouchterlony gel diffusion analysis established that PAb280 was an immunoglobulin of the immunoglobulin G1 subclass.

Immunoprecipitation analysis and Western blot analysis. We further analyzed the specificity of PAb280 in both immunoprecipitation and Western blot procedures. In the presence of goat anti-mouse immunoglobulin, PAb280 specifically immunoprecipitated a band of 17,000 molecular weight from extracts of wild-type SV40-infected cells (Fig. 3). This band comigrated with the small t band precipitated by PAb419. Unlike PAb419, however, PAb280 did not immunoprecipitate SV40 large T. Furthermore, in the immunoprecipitation of *dl* 883-infected cell extracts, PAb419 clearly precipitated large T but not small t, whereas PAb280 did not immunoprecipitate anything.

By the Western blot procedure, PAb280 was shown to bind directly to the small t polypeptide rather than to any associated species. The antibody bound strongly to a 17-kilodalton band present only in wild-type SV40-infected CV1 extracts but absent from *dl* 883-infected or uninfected CV1 cell extracts (Fig. 4B). The PAb419 control (Fig. 4A) clearly reacted with large T in both infected cell extracts and to small t in the wild-type SV40 but not in the *dl* 883-infected

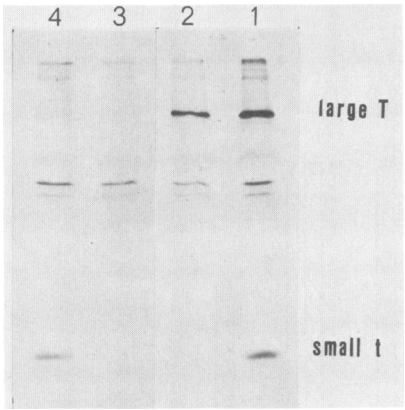

FIG. 3. Immunoprecipitation of small t from SV40-infected CV1 cells with PAb280. CV1 cells (10^6) were infected with wild-type SV40 or *dl* 883 virus and labeled with [^{35}S]methionine, and cell extracts were prepared at 72 h postinfection as described in the text. Lane 1, Wild-type virus-infected cell extract immunoprecipitated with PAb419 and goat anti-mouse immunoglobulin serum; lane 2, *dl* 883 virus-infected cell extract immunoprecipitated with PAb419 and goat anti-mouse immunoglobulin serum; lane 3, *dl* 883 virus-infected cell extract immunoprecipitated with PAb280 and goat anti-mouse immunoglobulin serum; lane 4, wild-type virus-infected cell extract immunoprecipitated with PAb280 and goat anti-mouse immunoglobulin serum.

cell extracts. PAb419 has previously been shown to cross-react with a 36,000-molecular-weight host protein (4). This reaction is clearly detected with the uninfected cell extract but is not detected in the infected cells, although the earlier study, using immunoprecipitation of labeled cell extract, has shown the host protein to be synthesized during lytic infection. This is a provocative observation which we are currently investigating further.

The fact that PAb280 recognizes small t in native form, as demonstrated by the immunoprecipitation, and also in Western blots, means that the reagent will be particularly useful for further analysis of small t.

Immunocytochemical localization of small t in lytically infected cells. We have performed an extensive series of experiments directed towards localizing small t in infected cells by using the PAb280 reagent. Wild-type SV40-infected CV1 cells, *dl* 883-infected CV1 cells, and mock-infected CV1 cells were stained by using the immunoperoxidase method with both PAb280 and PAb419 at various times after infection. PAb280 did not stain uninfected CV1 cells (data not shown) or *dl* 883-infected cells (Fig. 5B); no trace of reaction could be detected even under the highly sensitive conditions we developed with prolonged incubations and the use of the immunoperoxidase rather than the immunofluorescence technique. PAb419 also failed to stain uninfected CV1 cells (data not shown) but gives an intense nuclear stain on *dl* 883-infected cells (Fig. 5D) and on wild-type SV40-infected cells (Fig. 5C). When used to stain wild-type SV40-infected cells, PAb280 shows a clearly positive reaction with staining of both the nucleus and the cytoplasm readily apparent (Fig. 5A). The nuclear stain showed a different localization from that seen with PAb419. Although PAb419 staining of the nuclei was readily detected by 15 to 20 h postinfection and remained at a maximum level after 30 to 40 h postinfection, the PAb280 staining did not appear until 40 h and reached maximum intensity very late in infection (72 to 80 h). Even at these very late time points, the intensity of stain varied considerably from cell to cell. At these very late times, most of the cells showed a considerable cytopathic effect. Importantly, when PAb280 and PAb419 were mixed together and used to stain wild-type SV40-infected cells at 72 h postinfection (Fig. 6), stain was apparent intensively in the nucleus but was also clearly present in the cytoplasm.

DISCUSSION

PAb280 is the first monoclonal antibody to be isolated that recognizes the small t protein of SV40 and not large T. As such it represents a unique tool to investigate the properties of small t, particularly in situations when large T is also present in the material under investigation. The specificity of PAb280 was established in immunoprecipitation of [^{35}S]methionine-labeled extracts from CV1 cells infected with either wild-type SV40 or *dl* 883 mutant. It is clear that PAb280 efficiently immunoprecipitated small t but not large T from extracts of wild-type SV40-infected cells even though large T was present in the cell extract in a form accessible to PAb419. No protein specifically reacting with PAb280 was found in the deletion mutant-infected cells. Importantly, PAb280 also worked very efficiently in the Western blot procedure, allowing unique and ready detection of small t in both lytically infected and transformed cells (manuscript in preparation). Since in this latter technique total cellular proteins were analyzed rather than a soluble fraction, the specificity of PAb280 was very firmly established.

Immunocytochemical staining of wild-type and deletion mutant-infected cells with PAb280 gave some striking results. Strong staining with PAb280 was not detected until

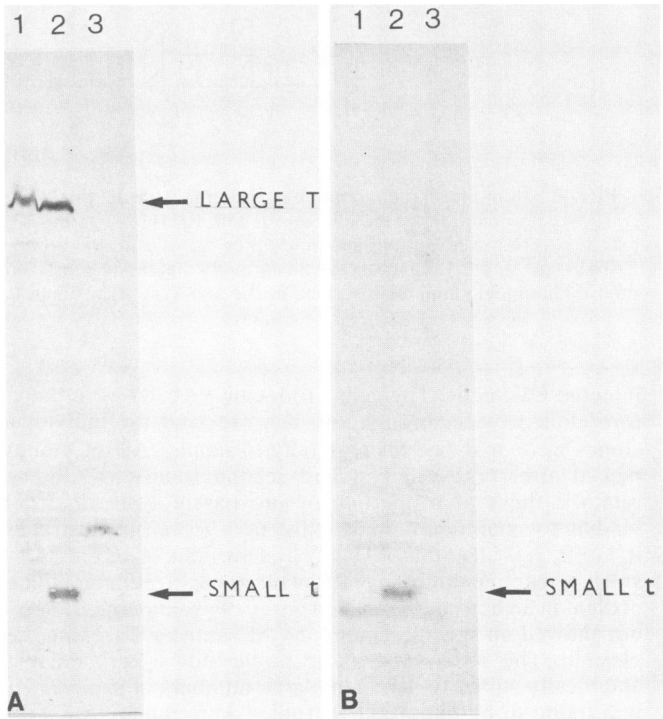

FIG. 4. Western blot analysis demonstrating the specificity of PAb280. CV1 cells (10^6) were infected with *dl* 883 virus (lane 1) or wild-type SV40 (lane 2) or mock infected (lane 3) and extracted in 200 μl of sample buffer. After sonication, 30 μl of the sample was run in each track. Blotting and antibody incubations were as described in the text. Iodinated molecular weight markers were used. Lane 1, extract of *dl* 883-infected cells; lane 2, extract of wild-type-infected cells; lane 3, extract of mock-infected cells. In (A) the blot is developed with PAb419 and in (B) it is developed with PAb280.

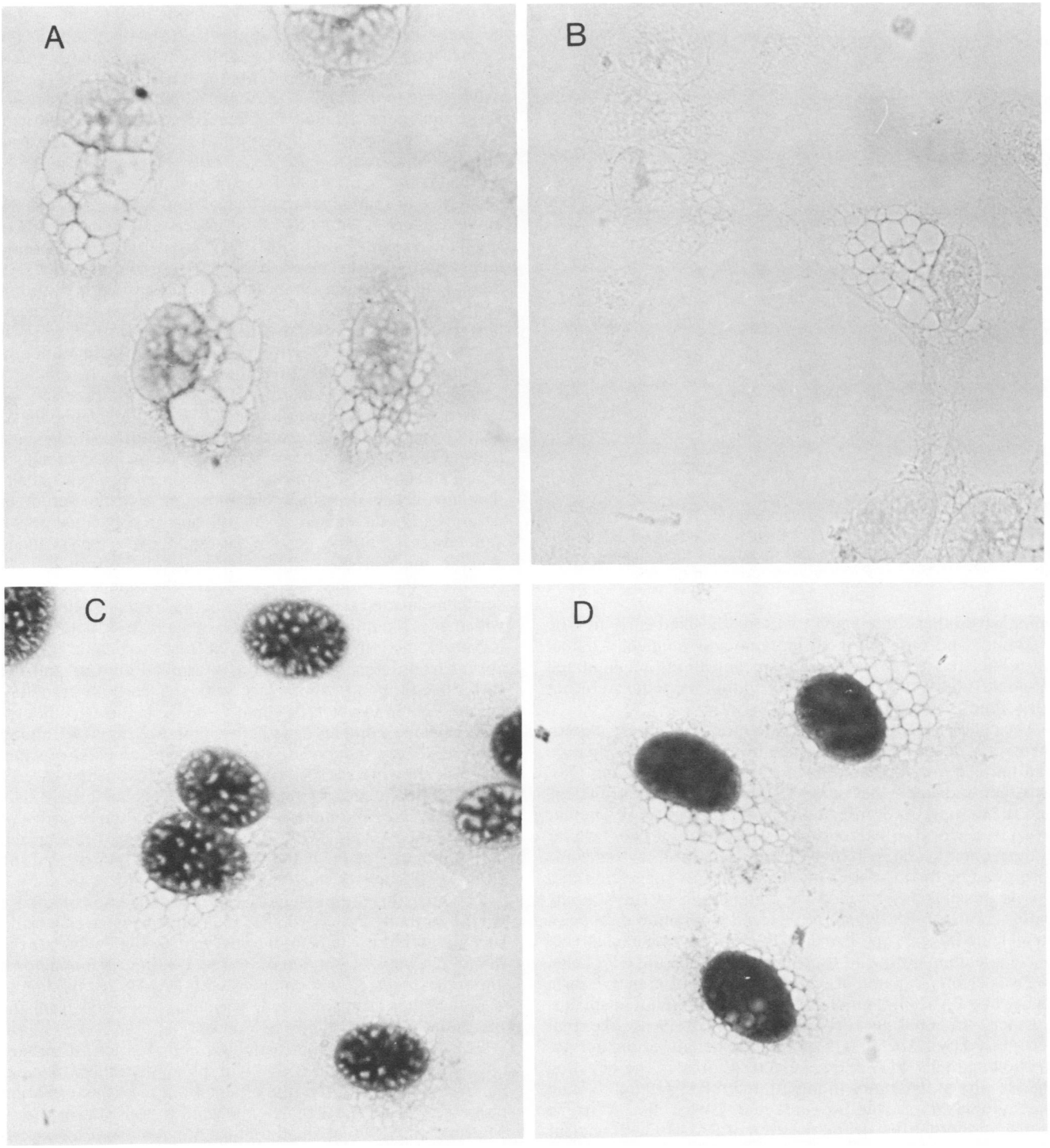

FIG. 5. Immunocytochemical localization of small t. CV1 cells infected with wild-type SV40 (A and C) or *dl* 883 virus (B and D) were fixed at 72 h postinfection and stained either with PAb280 (A and B) or PAb419 (C and D) by using the immunoperoxidase procedure described in the text. The stained cells were photographed with a Zeiss photomicroscope 3 and bright-field optics.

quite late in the lytic cycle, and the intensity of staining increased strongly at very late times in infection, i.e., between 60 and 72 h. This late onset of small t accumulation coincided with a switch in the rate of small t synthesis relative to that of large T that had been observed earlier (6). The observation may imply that small t plays some specific role late in the lytic cycle, but it could perhaps more simply reflect a disruption of the splicing apparatus at very late times, such that the precursor RNA is more readily spliced to remove the intron in the small t rather than in the large T message. The small t splice apparently is a thermodynamically favored reaction (1). Preliminary Western blot analysis of the stages of the lytic cycle supports the late accumulation of small t (data not shown). It should be

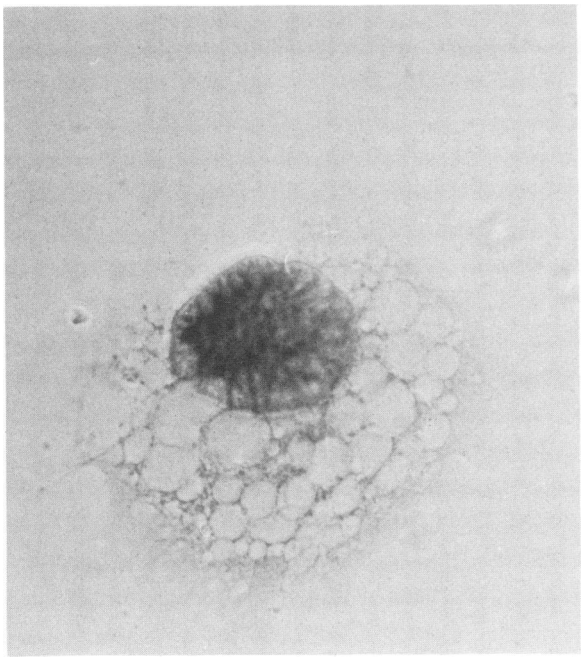

FIG. 6. Simultaneous staining of small t and large T. CV1 cells were infected with wild-type SV40, fixed, and then stained with a mixture of PAb280 and PAb419 as described in the text.

emphasized that some small t was synthesized early in lytic infection, and our failure to immunocytochemically detect this either reflects the very low concentrations accumulated or could imply that the PAb280 epitope is sequestered at early times of infection.

The cellular location of small t determined in these experiments was also unexpected since biochemical fractionation had implied a cytoplasmic localization for the protein (28). Our data indicated that small t resides in both the nucleus and the cytoplasm of infected cells. It is of interest whether small t performs distinct functions in these two cellular compartments and whether the nuclear and cytoplasmic forms can be distinguished biochemically. A suggestion that this is possible is provided by comparison of the staining patterns of PAb419, which recognized a common epitope of small t and large T, and PAb280. PAb419 gave an exclusively nuclear stain in both wild-type and *dl* 883-infected CV1 cells, yet was clearly capable of recognizing small t in native form since, like PAb280, it efficiently immunoprecipitated small t. Further, the epitope recognized by PAb419 is like that recognized by PAb280; it is resistant to denaturation, surviving boiling in 2% SDS and gel electrophoresis. The question then is why at late times in infection did PAb419 fail to stain the cytoplasm of wild-type-infected CV1 cells. A trivial explanation, that the strong reaction of PAb419 with nuclear large T in some way chelated all of the PAb419 antibody or the second detecting antibody can be ruled out. Careful determination of PAb419 titers by the immunoperoxidase test showed the antibody to be in gross excess, since dilution of the hybridoma supernatant by a factor of 1/100 still gave as intense a staining reaction. Chelation of the second antibody can be dismissed since, as shown, in Fig. 6, when PAb280 and PAb419 were used together, both the strong nuclear stain of PAb419 and the cytoplasmic stain of PAb280 were clearly visible.

Two possible explanations can be put forward to account for these findings, either there is a new molecular species produced in lytic infection and located in the cytoplasm that bears the PAb280 epitope but not the PAb419 epitope or the cytoplasmic form of small t adopts some particular conformational structure that prevents the binding of PAb419. We will be able to distinguish these possibilities by cell fractionation and more detailed biochemical studies. PAb280 can provide the basis for a quantitative assay of small t in infected and transformed cells, and this will allow us to correlate levels of small t expression with the biological properties of individual transformed cell lines. Such quantitative data are essential for a true understanding of the role of small t in transformation. PAb280 should also prove useful in investigation of the biochemical properties of small t. Although, in our initial immunoprecipitations with the antibody, we have failed to detect the two small t-associated host proteins described by Rundell (22), further work is necessary, since our conditions for washing the immunoprecipitates may have disrupted the complex.

Experiments to localize the PAb280 epitope precisely on small t, with deletions in the range of 0.54 to 0.59 map units, are in progress. Initial results indicated that the site lies just beyond the carboxy side of the 82 amino acids common to small t and large T. The only other antibodies specific to small t that have been described are those of hamster serum as described by Greenfield et al. (9) and a polyclonal rabbit antibody to a synthetic peptide corresponding to the six amino acids at the extreme carboxy terminus of the protein (12). Neither of these studies has reported on the cellular location of small t in infected cells, and so it is hard to compare our results with theirs. If our initial localization of the PAb280 determinant is correct, then small t can display at least two unique epitopes absent from large T, the carboxy-terminal epitope and the PAb280 epitope. It is unclear why the carboxy-terminal-unique 92 amino acids of small t should be so very poorly immunogenic compared with the amino-terminal 82 amino acids of shared sequence. Other examples do exist of small proteins showing such behavior, and at least in the case of the 27-amino-acid hormone glucagon (15), evidence exists that the area of the molecule recognized by the majority of the antibodies synthesized is discrete from that recognized by the majority of the helper T cells. It may thus be that the carboxy terminus of small t is preferentially recognized by T cells. Our efforts to present the carboxy terminus of small t via the antibody PAb419 do not seem to have been especially successful, but it will be of interest to determine whether the many hundreds of anti-small t-large T-common monoclonal antibodies induced will compete with PAb419 for binding to T or whether that epitope at least was excluded from the repertoire of the antibody response.

PAb280, in conjugation with the construction of viruses only producing small t (2), will hopefully allow a clear picture to emerge of the role of small t in the transformation process. We are particularly intrigued by the suggestion of Martin et al. (18) that small t may act in a manner analogous to known peptide growth factors, and we are now using the antibody to investigate that hypothesis.

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